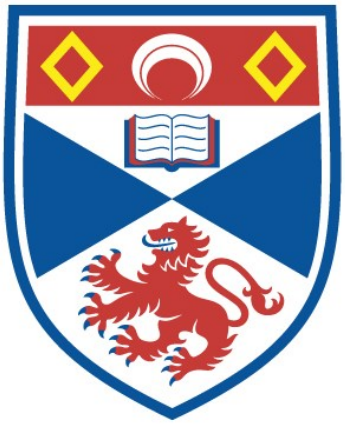
NEOPLASTIC TRANSFORMATION OF HUMAN THYROID EPITHELIAL CELLS BY IONIZING RADIATION

Zdenko Herceg

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



1996

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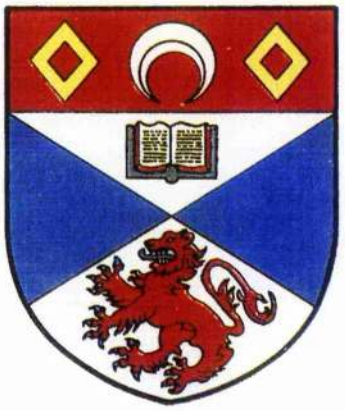

SCHOOL OF BIOLOGICAL AND MEDICAL SCIENCES

UNIVERSITY OF ST. ANDREWS

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Declaration

I, Zdenko Herceg, hereby certify that this thesis has been composed by myself, that it is a record of my own work, and that it has not been accepted in partial or complete fulfilment of any other degree or professional qualification.

Signed Zdenko Herceg Date

I was admitted to the Faculty of Science of the University of St. Andrews under Ordinance General No. 12, and as a candidate for the degree of Ph.D. on 1st October, 1992.

Signed Zdenko Herceg Date

Certificate

I hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate to the Degree of Ph.D.

Signature of Supervisors Date

Dr. Andrew C. Riches

Dr. Peter E. Bryant

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Last but not least, my wife and sons deserve special thanks, not just for putting up with my late nights, busy weekends, and no holidays but for the fun and generous spirit they bring to work every day.

Abbreviations

Ad12-SV40: hybrid of adenovirus 12 and simian virus 40

AIG: anchorage independent growth

AML: acute myelocytic leukaemia

ANCOVA: two way analysis of covariance

APS: ammonium persulphate

AP site: apurinic/apyrimidine site

ATP: adenosine 5'-triphosphate

BALB/3T3: mouse embryo fibroblast cell line

BEP2D: papilloma virus-immortalized human bronchial epithelial cells

BLOTTO: bovine lacto transfer technique optimizer solution

bp: base pairs

BSA: bovine serum albumine

BSAPBS: bovine serum albumine in phosphate-buffered saline

Bst NI: restriction enzyme recognizing and cleaving the sequence CCTGG

C3H 10T1/2: mouse embryo fibroblast cell line

Carnoy's fixative: 1 part glacial acetic acid : 3 parts absolute methanol

CM1: secondary anti-p53 antibody

CML: chronic myelogenous leukaemia

CTP: cytidine triphosphate

DAB: 3',3-diaminobenzidine

Dalton Mark VII-L: unstained protein molecular weight marker [Sigma]

dATP: 2'-deoxyadenosine 5'-triphosphate

dCTP: 2'-deoxycytidine 5'-triphosphate

DDT: dithiothereitol

DEPC: diethyl pyrocargonate

df: degrees of freedom

dGTP: 2'-deoxyguanosine 5'-triphosphate

DMSO: dimethyl sulphoxide

dNTPs: deoxyribonucleoside triphosphates

DO-1: primary anti-p53 antibody which precipitates both wild type and mutant p53 protein

dsb: double strand break

DTT: Dithiothreitol

dTTP: deoxythymidine

EcoR I: restriction enzyme recognizing the nucleotide sequence GAATTC

EDTA: ethylenediaminetetra-acetic acid

FCS: foetal calf serum

F12/DMEM: Ham's F12/Dulbecco MEM medium

G418: biochemical marker

Gel Soak 1: 1 0.5 N NaOH and 1 M NaCl

Gel Soak 2: 0.5 M Tris-HCl pH 7.4, and 3 M NaCl

GTE: glucose, Tris-HCl, and EDTA solution

GTP: guanosine triphosphate

H185B5: human mammary epithelial cells

Hae III: restriction enzyme recognizing the nucleotide sequence GGCC

HBS: Hank's balanced solution

HCT116: human colon carcinoma cell line

H&E: hematoxylin and eosin

hgprt: hypoxanthine-guanine phosphoribosyl transferase gene

Hind III: restriction enzyme recognizing the nucleotide sequence AAGCTT

Hoechst 33258 dye: DNA-intercalating dye which excites at 360 nm and emits at 490 - 500 nm

HOS: human osteocarcinoma cell line

Hph I: restriction enzyme recognizing and cleaving the sequence GGTGA

HPV: Human Papilloma virus

HRP: Horderadish peroxidase

HTori3: SV40 immortalized human thyroid epithelial cell line

HUC: immortalized human uroepithelial cells

HuT: series of malignant human fibroblast cell lines

IF8: anti-retinoblastoma mutant protein antibody

INT: 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-tetra-zolium chloride

KD: human diploid skin fibroblasts

KMS-6: normal human fibroblasts

KMST-6: γ-ray immortalized human fibroblast cell line

Lambda DNA: DNA ladder marker cut with restriction enzyme *EcoR I*

LB broth: Luria Broth consisting of Bactotryptone, Bacto yeast extract, NaCl, Bacto-agar, and ampicillin

LET: linear energy transfer

MNF116: murine anti-cytokeratin antibody [DAKO]

MNNG: 4-nitroquinoline-1-oxide and N-methyl-N'-nitro-N-nitrosoguanidine

MQ: Milli Q distilled water

Msp I: restriction enzyme recognizing and cleaving the sequence CCGG

NET buffer: lysis buffer consisting of 50 mM NaCl, 50 mM Tris, 5 mM EDTA, 1% NP-40, and 350 μg/ml phenylmethylsulfonyl fluoride

Nick stop mix: mixture of of 0.9% blue dextran, 0.03% bromocresol purple, and 20 mM EDTA

NIH 3T3: mouse embryo fibroblast cell line

NP-40: nonionic detergent Nonidet P40

nude mice: congenitally athymic nude mice, strain MF1 nu/nu [OLAC Ltd]

OH•: hydroxyl radical

P161: rabbit anti-mouse IgG HRP conjugated polyclonal antibody [DAKO]

PAb 240: monoclonal anti-p53 antibody which precipitates mutant p53 protein

PAb 405: murine monoclonal anti-T antigen antibody

PAGE: nondenaturing polyacrylamide gel

PBS: phosphate-buffered saline

PCR: polymerase chain reaction

PCR-SSCP: polymerase chain reaction - single strand conformation polymorphism analysis

^{32}P CTP: ^{32}P-labeled cytidine triphosphate

PE: plating efficiency

PEG: polyethylene glycol

Ph chromosome: Philadelphia chromosome (9;22 translocation)

PMSF: phenylmethylsulfonyl fluoride

primers: short oligonucleotide sequences

pSPT 18: vector containing RNApolymerase promoters from T7 and SP6 phages flanking the pUC 18 multiple cloning site

pSPT 19: vectors containing RNApolymerase promoters from T7 and SP6 phages flanking the pUC 18 multiple cloning site

Rb: retinoblastoma gene

RBE: relative biological effectiveness

RCF: relative centrifugal force

REACT 2: reaction buffer consisting of 50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, and 50 mM NaCl [Gibco BRL]

REACT 3: reaction buffer consisting of 50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, and 0.1 M NaCl [Gibco BRL]

RFLP: restriction fragment lenght polymorphisms

RHEK-1: human epidermal keratinocyte cell line

rpm: revolutions per minute

RPMI 1640: Rosewell Park Memorial Institute tissue culture medium 1640

SDS: sodium dodecyl sulfate

SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electophoresis

SEM: scanning electron microscopy

SF: surviving fraction

S081-201: sheep anti-mouse polyclonal antibody conjugated with Horderadish peroxidase [SAPU]

ssb: single strand break

SSC: buffer consisting of 1.5 M NaCl and 0.15 M sodium citrate

SSPBS: sheep serum in phosphate-buffered saline

SV40: Simian virus 40

SV ori$^-$: replication origin defective SV40 genome

SW480: human colon adenocarcinoma cell line

T24: human bladder carcinoma cell line

T7: bacteriophage RNA polymerase

T-antigen: SV40 large T antigen (94 kDa)

t-antigen: SV40 small t-antigen (17 kDa)

Taq: thermostable DNA polymerase isolated from eubacteria *Thermus aquaticus*

TBE: Tris-borate and ethylenediaminetetra-acetic acid

TE: Tris (hydroxymethyl) methylamine and ethylenediaminetetra-acetic acid solution

TEMED: N,N,N',N' - tetramethylethylenediamine

TNE: Tris Cl, NaCl, and ethylenediaminetetra-acetic acid solution

TPA: diterpene phorbol ester, and 12-O-tetra-decanoylphorbol-13-acetate

Tris: Tris (hydroxymethyl) methylamine

UTP: uridine 5'-triphosphate

UV: ultraviolet light

VP1-3: SV40 viral capsid protein

ΦX174 Hae III digest: DNA marker

Abstract

Neoplastic transformation of human thyroid epithelial cells has been investigated following exposure to ionizing radiation *in vitro*. The effects of radiation type, irradiation regime, and postirradiation passaging were examined using a human thyroid epithelial cell line, designated HTori3, which was previously immortalized with SV40 genome.

Exponentially growing HTori3 cells were irradiated with graded doses of ^{137}Cs γ- and ^{238}Pu α-irradiation. Cells were irradiated with either a single or multiple doses of 0.5, 1, 2, 3, or 4 Gy γ-radiation, or single doses of 0.125, 0.25, 0.5, 1, or 1.5 Gy α-radiation. Following passaging, the cells were transplanted into the athymic nude mice, and the animals were screened for tumour formation.

Statistically significant increases in tumour incidence were obtained with both γ- and α-irradiation and with both single and multiple irradiation regimes as compared with the un-irradiated group. Regardless of radiation type and or radiation regime there appears to be a trend, with increasing doses of radiation, in which tumour incidence increases and reaches a maximum, after which the tumour incidence decreases. Tumours were characterized by histopathological examination as undifferentiated carcinomas. Investigation of expression time following irradiation demonstrated that post-irradiation passaging, generally regarded as a critical step for expression of radiation-induced DNA damage, was not a prerequisite for the neoplastic conversion of irradiated cells with this system.

Cell lines were established from the tumours and their identification and characterization carried out. All cell lines established

were determined to be derived from the parent HTori3 cells by DNA fingerprinting, karyotype analysis, cytokeratin staining, and SV40 large T-antigen staining.

Tumorigenicity of the cell lines was confirmed by retransplantation. Comparison of the morphology *in vitro* showed that the tumour cell lines retained the basic epithelial morphology of the parent HTori3 cells. Investigation of radiosensitivity showed that none of the 6 tumour cell lines examined had a higher radiosensitivity compared to the parent HTori3 cells. This excludes the possibility that the observed transformation was the result of the selection of a pre-existing transformed subpopulation of the parent cells but that radiation-induced transformants were being induced *de novo*.

The tumour cell lines were screened for mutations in H- and K-*ras* oncogenes using restriction enzyme analysis of PCR amplified DNA. No mutations were detected in 26 tumour cell lines suggesting that mutations in these two genes do not appear to be involved in radiation-induced neoplastic transformation in human thyroid epithelial cells. Screening for mutations in p53 protein using immunoprecipitation method detected no mutations in 6 tumour cell lines.

This human thyroid epithelial cell line may thus be useful for the *in vitro* study of cellular and molecular mechanisms that are involved in human epithelial cell carcinogenesis.

"There is an incidence of cancer that cannot be reached by the physician's medicaments, surgeon's knife, or any organized advice against panic. Nothing but the actual conquest of cancer itself will remove this sword that today hangs over every head."

From the welcome by President Glenn Frank to participants in "A Symposium on Cancer" Madison, Wisconsin, 1936

Chapter 1

INTRODUCTION

1.1. General introduction

The study of human cell transformation *in vitro* by carcinogenic agents is of particular importance for an improved understanding the molecular and cellular mechanisms underlying human carcinogenesis. Over the last two decades, there has been considerable interest in the study of *in vitro* neoplastic transformation by ionizing radiation.

Ionizing radiation was the first environmental agent shown to be mutagenic (Muller, 1927) and is now well recognized as a carcinogen (Upton, 1984; Shigematsu & Kagan, 1986). The carcinogenic actions of ionizing radiation has been well documented in epidemiological studies of humans (BEIR, 1972; Rossi & Kellerer, 1974; UNSCEAR, 1977). Background irradiation (from cosmic rays and radioisotopes) and modern medicine makes ionizing radiation a common carcinogenic agent to which humans are exposed (Breimer, 1988).

It has been shown that ionizing radiation can induce tumours in various tissues and organs in animals (Upton, 1986) and can cause

transformation of rodent cells in culture (Borek & Sachs, 1966; Borek & Hall, 1973, Borek, 1982; Little, 1986).

Although there are many reports where *in vitro* transformation of rodent cells has occurred following irradiation, there are only a few reports of *in vitro* transformation of human cells. In particular, the literature concerning the transformation of human epithelial cells *in vitro* is scarce. This can be attributed partly to the difficulties in growing human cells in culture, especially those of epithelial origin, and to the relatively stable phenotype of human cells (Chang, 1986). Thus, the mechanism underlying neoplastic transformation of human cells is poorly understood.

Since the most common human cancers develop from epithelial cells the study of neoplastic transformation of such cells is of great importance. The majority of studies of carcinogenesis have relied upon the use of rodent cells. In designing experimental models to study mechanisms of human carcinogenesis it can be argued that it would be more relevant to use human epithelial cells.

In this study an SV-40 immortalized human thyroid epithelial cell line was used in order to develop an *in vitro* experimental model for easy induction and detection of neoplastic transformation by ionizing radiation.

1.2. Mechanisms of radiation transformation

Ionizing radiation has long been implicated in the induction of tumours in humans and experimental animals (Kaplan, 1967). The carcinogenic effect of radiation is thought to be due to DNA alteration or the failure of/inaccuracy of subsequent DNA repair mechanisms.

1.2.1. DNA damage induced by ionizing radiation

It is now almost certain that DNA represents the most critical target in mammalian cells that must be damaged to kill the cell (Hall, 1988). Radiation-induced DNA damage can lead to cell killing, mutation induction in specific genes, or carcinogenesis. To study radiation carcinogenesis, it is essential to understand the events which are involved at the molecular level.

Although the literature on the genetic effects of ionizing radiation is immense, the molecular mechanism(s) involved are not clearly understood. One reason for this is that radiation produces a large number of DNA lesions (Hutchinson, 1985). These include a variety of base alterations, DNA single- and double-strand breaks, DNA deletions, and inversions (Wallace, 1983; Hutchinson, 1985; Urlaub *et al.*, 1986).

The type of DNA damage depends on the type of radiation to which biological material is exposed. High energy transfer (LET) radiation (α-particles, protons, heavy charged ions) is believed to interact predominantly directly with the critical targets in the cells (Hall, 1988). This is called the direct action of radiation. Individual high LET radiation particles have sufficient kinetic energy to directly disrupt the structure of critical molecules producing the chemical and biological changes (Figure 1.2.1.1.).

Alternatively, low LET radiation (γ- or X-rays) exerts its effect to a greater extent by the so called indirect action. This involves interaction of radiation with other atoms and molecules in the cells to produce free radicals which can diffuse short distances and damage the cellular DNA (Figure 1.2.1.1.). The proportion of indirect damage of the overall damage

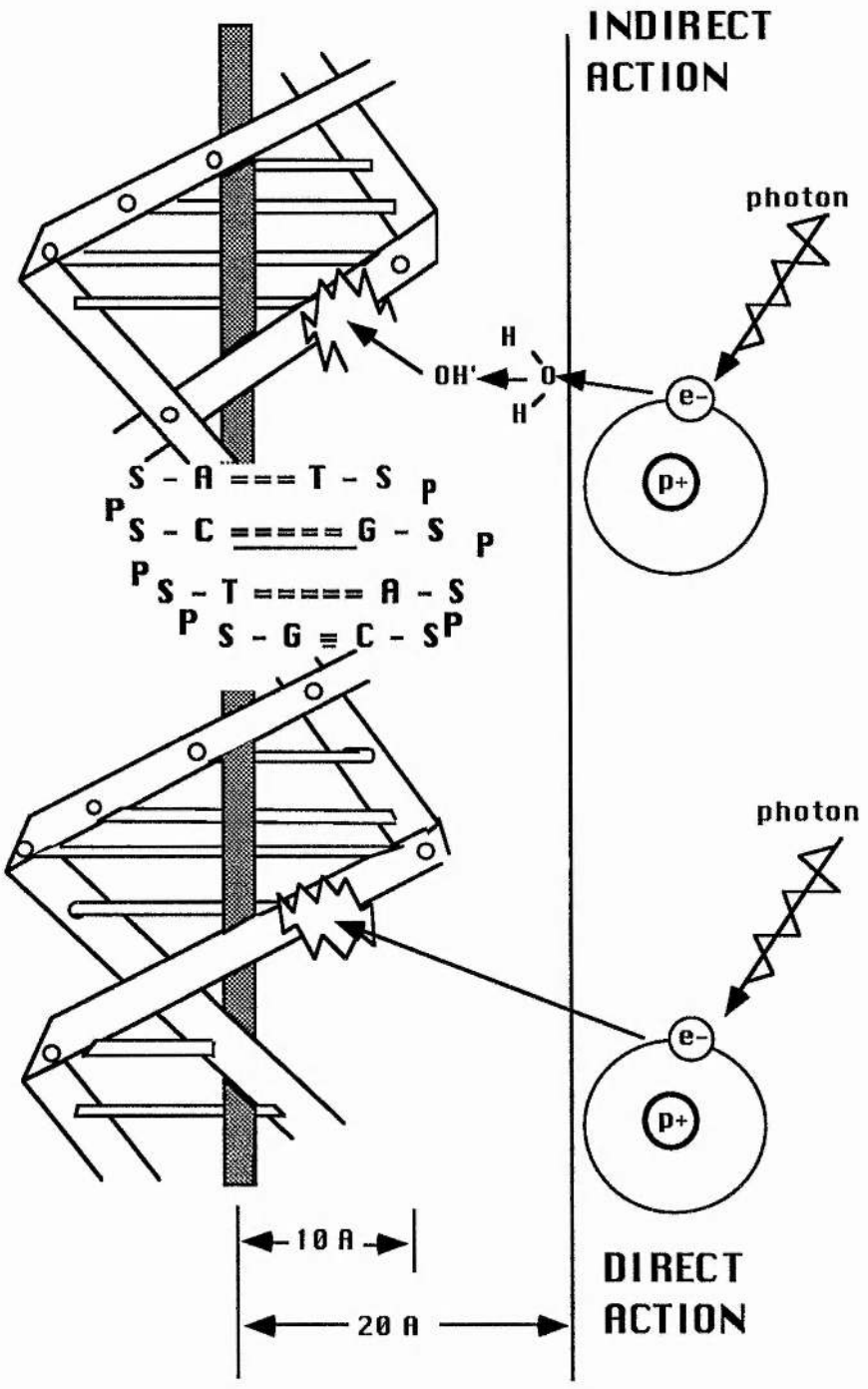


Figure 1.2.1.1. Mechanisms of radiation-induced DNA damage: the direct and indirect actions of ionizing radiation. The letters S,P,A,T,G, and C represent sugar, phosphorus, adenine, thymine, guanine, and cytosine, respectively. Indirect action is dominant for sparsely ionizing radiation, such as X-rays (redrawn from Hall, 1988).

is greater following low LET radiation compared to high LET radiation (Milligan *et al.*, 993a; 1993b).

Since 80% of a cell is composed of water, it is believed that cellular water plays a critical role in the production of free radicals (Hall, 1988). Interaction of radiation with water molecules results in excitation and ionization of these molecules. This is called the physical phase which takes only 10^{-15} sec. Water ions produced in this way have a very short half life of about 10^{-10} sec, they decay to form free radicals (half life about 10^{-5} sec). Among these, the highly reactive hydroxyl radical (OH•) is estimated to play major role in DNA damage induced by low LET radiation.

Hydroxyl radicals can diffuse very quickly and cause the breakage of chemical bonds of biologically important molecules. For istance, OH• radicals may oxidise DNA molecule abstracting hydrogen atoms from the base altering nucleotide structure and stability (Hutchinson, 1985).

The time scale involved in these events varies enormously. While the physics and chemistry of the process may take only a split second, biologicals effect (eg. cancer) may be expressed years to decades later.

Ionizing radiation exerts the bulk of its damaging effects through free radicals, particularly hydroxyl radicals (Breimer, 1988). There are broad spectrum of primary DNA lesions that are produced by ionizing radiation (Ljungquist & Lindhal, 1974; Ward & Kuo, 1976; Cerutti, 1976; Teoule & Cadet, 1978; Hutchinson, 1985; Breimer, 1985) (Figure 1.2.1.2.). The most common lesions are:

single strand break (ssb)
double strand break (dsb)
base damage
DNA protein cross-links
sugar damage

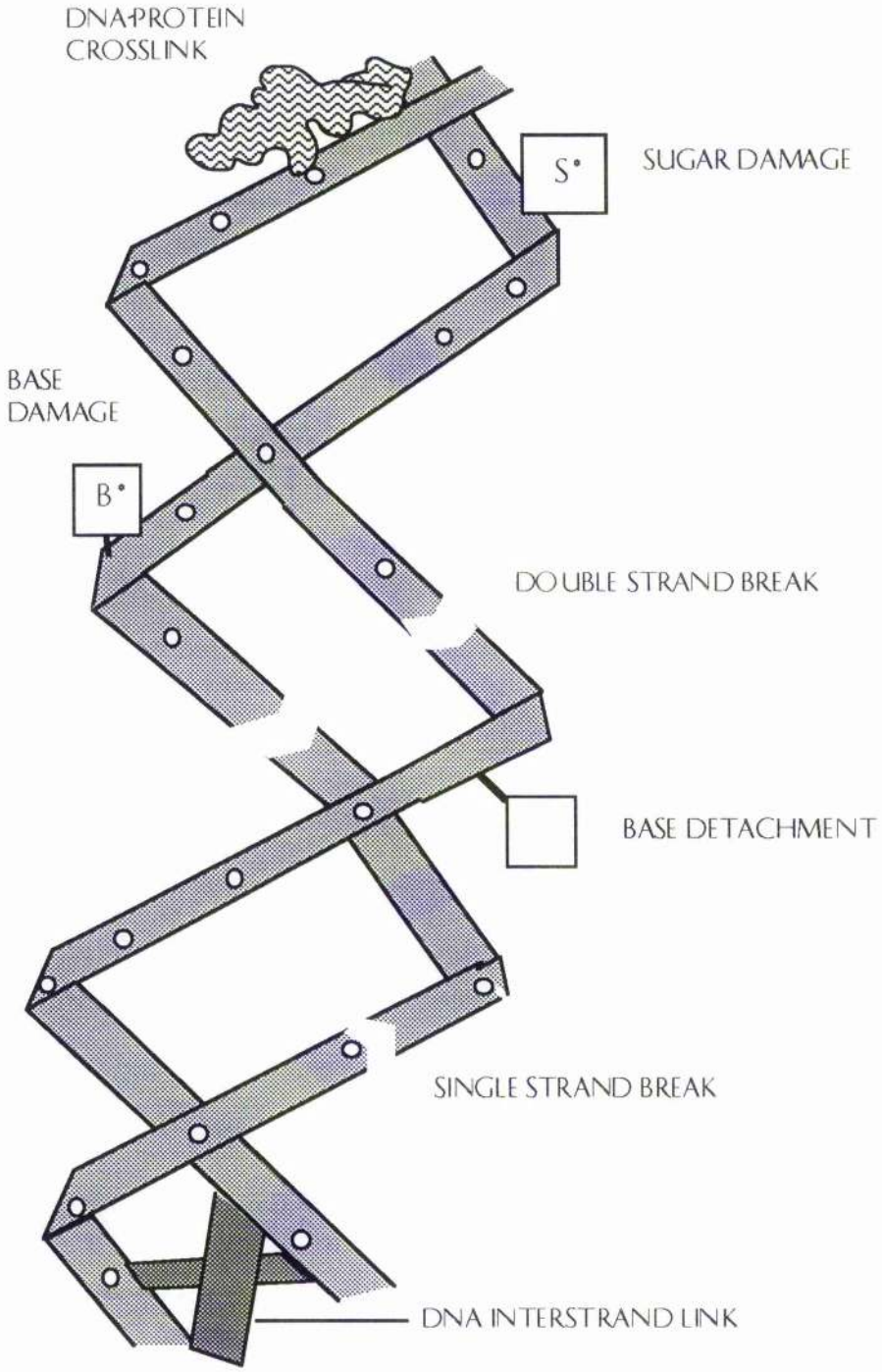


Figure 1.2.1.2. The spectrum of DNA damage that occurs as a result of radiation exposure (redrawn from Frankenberg-Schwager, 1989).

1.2.1.1. Single strand breaks

The literature on DNA strand breaks induced by ionizing radiation is immense (for reviews see Cerutti, 1976; Hutterman *et al.*, 1978; Thacker & Cox, 1983; Hutchinson, 1985; Thacker, 1986; Breimer, 1988). It is now accepted that both single strand breaks (ssb) and double strand breaks (dsb) are generated by direct action and indirect action (free radicals) of ionizing radiation (Breimer, 1988).

The ssb usually occur at the phosphodiester linkage of the DNA chain, and can be produced by direct action of radiation, as a result of a local deposition of relatively large amounts of radiation energy at the site of the phosphodiester linkage. However, low LET radiation produces ssb mainly through free radicals (particularly hydroxyl radical) (Milligan *et al.*, 1993b). The ssb can also be produced as a consequence of base and sugar damage.

1.2.1.2. Double strand break

A dsb is described as a breakage of both DNA strands at sites which are less then 3 base pairs apart (Bryant, 1984; 1988). The dsb is regarded to be one of the most important primary DNA lesions and responsible for chromosomal aberrations and carcinogenic effects of ionzing radiation (Thacker 1986; Bryant, 1989). There is evidence that the dsb induces mutations by the mechanism of DNA deletion (Whaley & Little, 1986; Helbig *et al.*, 1994).

The number of DNA breaks (ssb and dsb) is proportional to the radiation dose, and is a measure of the degree of insult, i.e. the total amount of radiation energy deposited (Coquerelle *et al.*, 1976; Breimer, 1988).

1.2.1.3. Base damage

Although the ssb have been traditionally regarded as the most frequent radiation lesion, it is estimated that base lesions are equally frequent (Hutchinson, 1985). According to some authors, base damage is one of the most common radiation induced DNA lesion (Cerutti, 1976; Frankenberg-Schwager, 1989). Ward (1985) has calculated that base lesions may be at least twice as common as DNA strand breaks.

There are many different types of base damage induced by ionizing radiation (Hutchinson, 1985). These include saturation and fragmentation of the purine and pyrimidine bases (Teoule *et al.*, 1977; Teoule & Cadet, 1978; Bonicel *et al.*, 1980; Breimer, 1984; Hutchinson, 1985; Cadet & Berger, 1985; Breimer & Lindahl, 1985a). Partial or complete destruction of purines and pyrimidines are thought to be caused mainly by the action of free radicals, particularly OH• radicals (Hutchinson, 1985).

1.2.1.4. DNA protein cross-links

Cellular DNA is normally linked to various nuclear proteins which are involved in packaging of DNA (histones), transcription (transcription factors) and replication proteins (Chiu *et al.*, 1982; Rawn, 1983; Adams *et al.*, 1986; Oleinick *et al.*, 1986; Alberts *et al.*, 1991). It has been demonstrated that ionizing radiation causes additional DNA-protein links in the V79-4 cell line (Oleinick *et al.*, 1986).

1.2.1.5. Sugar damage

Sugar molecules (2-deoxyribose), linked with phosphodiester, represents the backbone of the DNA molecule. Sugar damage may be the result of both the direct and indirect action of ionizing radiation. It is

thought that sugar damage may lead to the base loss and the ssb (Lamaire *et al.*, 1984; Deeble & von Sonntag, 1984).

1.2.2. DNA repair

DNA is a highly reactive chemical species and therefore is the target of numerous physical (eg. radiation) and chemical agents (some anticancer drugs). It is therefore quite remarkable that DNA is functionally more stable than the two other cellular macromolecules, RNA and protein. This stability can be attributed to the double-helical structure, which carries the information in duplicate. Equally important for functional stability of DNA are various DNA repair mechanisms.

Cells must repair DNA damage in order to maintain normal function and proliferation. Eukaryotic as well as prokaryotic cells possess a complex mechanism for repair of different types of DNA damage. Several mechanisms for DNA repair have been proposed such as direct repair, excision repair, recombinational repair, repair of crosslinks (for a review see Sancar & Sancar, 1988). For example, direct ligation and the single strand annealing models have been proposed as mechanisms for dsb (Lin *et al.*, 1984; 1987). A model for dsb repair involving DNA recombination was also proposed by Resnick (1976).

Different repair mechanisms may be activated depending on the type and extent of DNA damage. For example, following base damage, two modes of excision repair are known to occur: nucleotide excision repair and base excision repair (Sancar & Sancar, 1988). The nucleotide excision repair involves the removal of a section of DNA containing the lesion and the repair synthesis using the intact DNA strand as a template. This process seems to require at least four enzyme activities: a) a specific

endonuclease to cut the DNA strand close to the lesion; b) an exonuclease to remove the damage; c) a polymerase to synthesize DNA in the gap; and d) a ligase to restore the continuity of the DNA strand.

In base excision repair, on the other hand, the damaged base is enzymatically removed by a specific DNA glycosylase. The resulting apurinic/apyrimidine (AP) site is restored by the action of an AP endonuclease and exonuclease and the gap closed by repair synthesis and ligation.

Nucleotide excision repair acts on "bulky lesions" (eg. pyrimidine dimers), makes repair patches 10 to 20 nucleotide long and requires up to 24 hours for completion. Base excision repair acts on small lesions consisting at most of several nucleotides (eg. deaminated cytosine), and repair is complete within an hour.

Following radiation-induced DNA damage both modes of repair will be activated. "Short-patch" repair will operate on small DNA lesions, while "long-patch" repair will operate in response to "bulky" lesions (Hanawalt *et al.*, 1979; Teoule, 1987).

A deficiency in DNA repair appears to be a factor facilitating the accumulation in one cell of the gene changes required for development of cancer (Riley, 1982; Sanford *et al.*, 1989). It has been reported that human mammary epithelial cells can aquire a DNA repair deficiency spontaneously during prolonged culture or after treatment with *ras* oncogene (Sanford *et al.*, 1992). This repair deficiency was manifested as an abnormal frequency of persistent chromatid damage after exposure to X-rays during the G_2 phase of the cell cycle.

The DNA repair process should result in complete and error-free repair. Unrepaired or misrepaired DNA damage may be expressed in

different ways, such as chromosomal aberrations and micronuclei. These may consequently lead to cell death or neoplastic transformation.

1.2.3. Radiation induced mutations

A mutation is defined as a heritable change in the nucleotide sequence of a DNA molecule (Watson, 1973; Lewin, 1990). Although there are changes that produce no change in the amino acid sequence of the protein product (synonymous mutations), many changes lead to changes in protein product (non synonymous mutations).

Mutations may occur either as a result of an error in normal DNA replication or DNA damage (e.g. radiation-induced) which has been misrepaired. There is evidence that mutations are not randomly distributed in the genome, but that are sites where mutations occur with greater frequency (Watson, 1973; Lewin, 1983). These sites are called mutation "hotspots". It is suggested that "hotspots" exist within the genome for radiation induced changes (such as deletions) (Sankaranarayanan, 1991).

Deletions are defined as the loss of one or more nucleotide bases from the DNA molecules. In contrast, insertions are the addition of one or more nucleotide bases into the DNA sequence. It is believed that radiation induces predominantly the deletion type of mutation

Recently, it has been suggested that deletions may be the most common lesion induced by ionizing radiation (Waldren *et al.*, 1986). Studies with the hypoxanthine-guanine phosphoribosyl transferase gene (*hgprt*) have shown that 58 independent mutants of the gene induced by γ-radiation were the result of complete deletion of the gene (Cox & Masson, 1978).

1.2.4. Activation of cellular proto-oncogenes

Proto-oncogenes are normal evolutionary conserved cellular genes, present in all mammalian cells which, when activated, may contribute to the carcinogenic processes. Many of these genes have been found to have a counterpart among the viral oncogenes which led to the conclusion that viral oncogenes were mutated forms of essential cellular genes (Bishop, 1983). Proto-oncogenes encode proteins involved in all aspects of cell proliferation, and differentiation (for review, see Bishop 1983, Weinberg R., 1989; Wynford-Thomas, 1991; Minden & Pawson, 1992). In normal cells, these genes are expressed, but do not result in cancer development as their expression is tightly controlled.

Activation of proto-oncogenes usually occurs as the result of mutation, gene amplification, or rearrangements (for review, see Land *et al.*, 1983; Bishop, 1983). Single point mutations can significantly alter the biological function of normal genes. For example, *ras* genes acquire transformation-inducing properties following point mutation within their coding regions (Tabin *et al.*, 1982; Taparowski *et al.*, 1982). It was found that mutations in *ras* oncogenes were predominantly located in codons 12 (Tabin *et al.*, 1982; Reddy *et al.*, 1982), and 61 (Taparowski *et al.*, 1983; Yuasa *et al.*, 1983). It appears that the glycine residue at position 12 is essential for the normal function of *ras* proteins. Substitution of Gly12 by any other amino acid residue results in the oncogenic activation of these molecules (Seeburg *et al.*, 1984).

Amplification of a proto-oncogene can also contribute to the development of a malignant phenotype. An amplified proto-oncogene results in the over-production of normal gene products which, at higher

levels, can provide a selective advantage to the cell. Such events may be involved in the primary development of tumours or with subsequent changes associated with tumour progression. Amplification of c-*myc* was first reported in the HL-60 cell line derived from a patient with acute myelocytic leukaemia (AML) (Collins & Groudine, 1982). Significant increases (four to seven fold) in c-*myc* expression was observed in many chemically and radiation-transformed C3H 10T1/2 mouse embryo cell lines (Shuin *et al.*, 1986).

Another mechanism of proto-oncogene activation is gene rearrangement. A classic example of gene rearrangement is the Philadelphia (Ph) chromosome (9;22 translocation) which is associated with chronic myelogenous leukaemia (CML). Following genetic analysis of the site of translocation in the Ph chromosome it was found that the proto-oncogene c-*abl*, normally found on chromosome 9, was translocated to chromosome 22 and fused with the *bcr* gene (de The *et al.*, 1990). This change results in the production of an altered mRNA, and consequently an abnormal protein product, which has transforming ability. Thus, either qualitative (e.g mutation) or quantitative (e.g. overexpression), or both, abnormalities of proto-oncogenes can contribute to the development of neoplasia.

Although most *in vitro* studies have failed to identify a radiation-induced oncogene, several reports suggest that activation of proto-oncogenes by ionizing radiation may be important in tumour induction. The activation of cellular *ras* oncogenes have been detected in rodent tumours induced by ionizing radiation (Guerrero *et al.*, 1984a; 1984b; Sawey *et al.*, 1987). It was found that mouse lymphomas induced by γ-radiation contained an activated K-*ras* oncogene. Following molecular analysis, it was found (Guerrero *et al.*, 1984a) that the activated K-*ras* had a point

mutation in the second base of 12th codon. There are also other reports describing the activation of K-*ras* oncogenes, including strontium induced osteosarcoma in mice (Merregaert *et al.*, 1986), and plutonium induced lung carcinoma in dogs (Frazier *et al.*, 1986). Borek *et al.* (1987), however, reported that malignant transformation of hamster embryo and mouse 10T1/2 cells *in vitro* involved the activation of distinctive transforming genes which do not represent activated forms of the *ras* gene family, nor any of additional 9 oncogenes analysed. Molecular cloning and characterization of these transforming genes have not yet been done.

1.2.5. Inactivation of tumour suppressor genes

Tumour suppressor genes (also called recessive oncogenes or antioncogenes) have the opposite effect from oncogenes. When they are functioning normally they can restrict, or inhibit, cell proliferation. The loss of normal function of tumour suppressor genes as negative regulators of cell proliferation is believed to lead to tumour development. These genes are now emerging as major factors in the induction and progression of many human cancers. Several tumour suppressor genes have been discovered and isolated with the prospect of discovering many others in the future. The most studied tumour suppressor genes are p53 (Lane & Crawford, 1979), retinoblastoma (Rb) (Friend *et al.*, 1986; Fung *et al.*, 1987), and more recently p16 (Marx, 1994).

The p53 gene is the most widely mutated gene in human tumorigenesis (Hollstein *et al.*, 1991, Levine *et al.*, 1991). The p53 gene encodes a transcriptional activator (Fields & Jang, 1990; Farmer *et al.*, 1992; Kern *et al.*, 1992) whose targets may include genes that regulate genomic stability (Livingstone *et al.*, 1992, Yin *et al.*, 1992), cellular response to DNA

damage (Kastan *et al.*, 1992), and cell cycle progression (Michalovitz *et al.*, 1990).

One of the major molecular effects of radiation is the deletion of DNA sequences, which often results in cell death. However, cells which survive may have deletions in regulatory genes, such as the tumour suppressor genes. After examination of DNA and RNA from osteosarcomas and soft-tissue sarcomas, it was suggested that radiation-induced osteosarcomas may be the result of inactivation of the Rb gene following radiation treatment and that deletion of regulatory sequences may be part of the general mechanism of radiation-induced carcinogenesis (Weichselbaum *et al.*, 1988).

Additional support of this concept is the association of specific chromosome deletions observed within many human tumours following treatment with cytotoxic agents. Patients who develop acute non-lymphocytic leukaemia following radiotherapy and chemotherapy exibit specific chromosomal deletions (Rowley *et al.*, 1981; Le Beau *et al.*, 1986). Various radiation-induced deletions of chromosomal regions containing tumour suppressor genes in human bronchial epithelial cell lines were also reported (Willey *et al.*,1993).

It is interesting to note that Endo *et al.* (1993) found an alteration in the Rb gene which was associated with the immortalization of human fibroblasts (KMS-6) treated with γ-rays (Namba *et al.*, 1985). Restriction fragment length polymorphism analysis (RFLP) and Southern blot analysis revealed an alteration in chromosome 13q12-14 and structural abnormalities in the Rb gene itself. Furthermore, an abnormal Rb gene protein (pRb) was expressed in immortalized KMST-6 cells, whereas normal KMS-6 cells expressed the normal pRb (Endo *et al.*, 1993). The

authors suggested that inactivation of this gene may be one of key events of the immortalization of human cells.

1.3. Cancer as a multistep process

It is now widely accepted that malignant transformation of cells in culture, as *in vivo*, is a multistep process involving a series of genetic alterations and cell selection (Farber, 1984; Klein & Klein, 1985). This has been confirmed in a number of ways. Statistical analysis of age-incidence curves indicate that at least three successive mutation-like changes are required for leukaemias and at least six for carcinomas (Farber & Cameron, 1980).

Epidemiological studies have suggested that five or six independent steps are required for malignant transformation (Peto, 1977). More recently, using a model of colorectal carcinogenesis (Figure 1.3.1.), it was demonstrated that at least four genetic changes are required both in cellular proto-oncogenes and tumour suppressor genes for the aquisition of the malignant phenotype (Fearon & Vogelstein, 1990).

For some types of malignant neoplasia, such as colonic carcinoma, well-defined stages of carcinogenesis have been identified histologically and tissue have been isolated for genetic analysis (Vogelstein *et al.*, 1988). This has allowed comparision of cells from normal, polyp or carcinomatous tissue by cytogenetic and molecular methods.

Namba *et al.* (1988) proposed a multi-step model for neoplastic transformation of human fibroblasts (Figure 1.3.2.). It was suggested that γ-rays worked as an initiator, and the H-*ras* oncogene played a role in the progression.

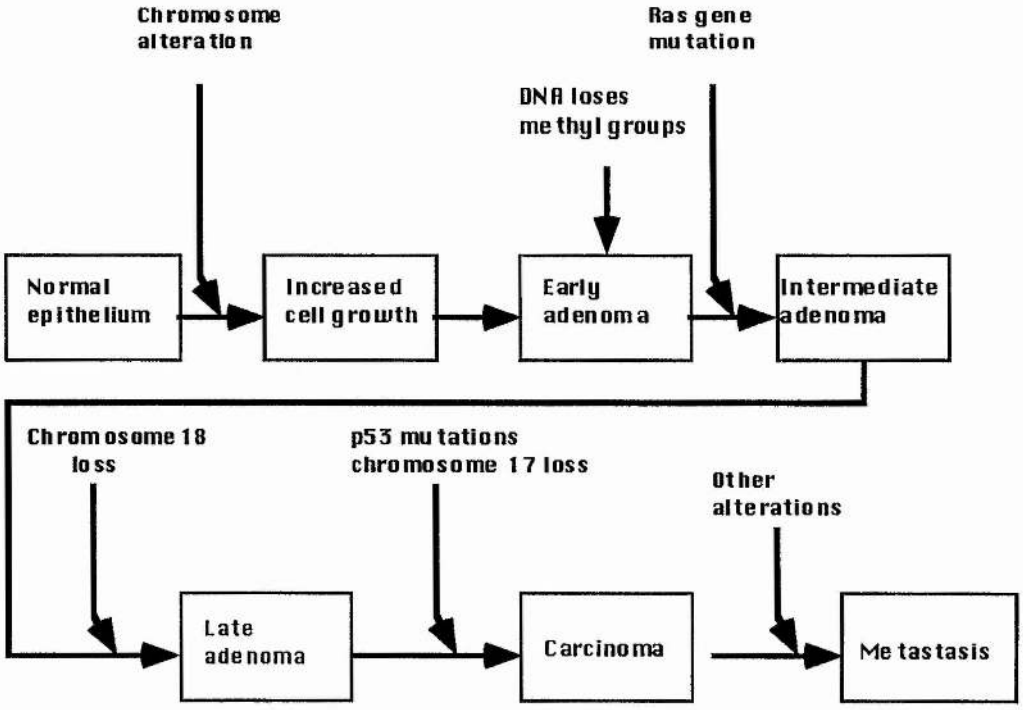


Figure 1.3.1. A molecular model for multi-stage tumorigenesis in the colon (redrawn from Fearon & Vogelstein, 1990).

Traditionally, different terms are used for the stages involved in carcinogenesis: initiation, promotion, and progression (Farber, 1984).

1.3.1. Initiation.

Initiation is usually a rapid and irreversible process. Strong evidence exists that changes in DNA are responsible for initiation. This was formulated early in terms of the induction of an altered base, miscoding lesion, and a mutation in the classical sense. Moreover, subsequent research in "gene dynamics" has indicated that the possibilities for other forms of initiation such as rearrangement of genes, the activation and modulation of gene expression, and gene amplification exist. Thus, an initiating agent is defined as a chemical, physical, or biological agent

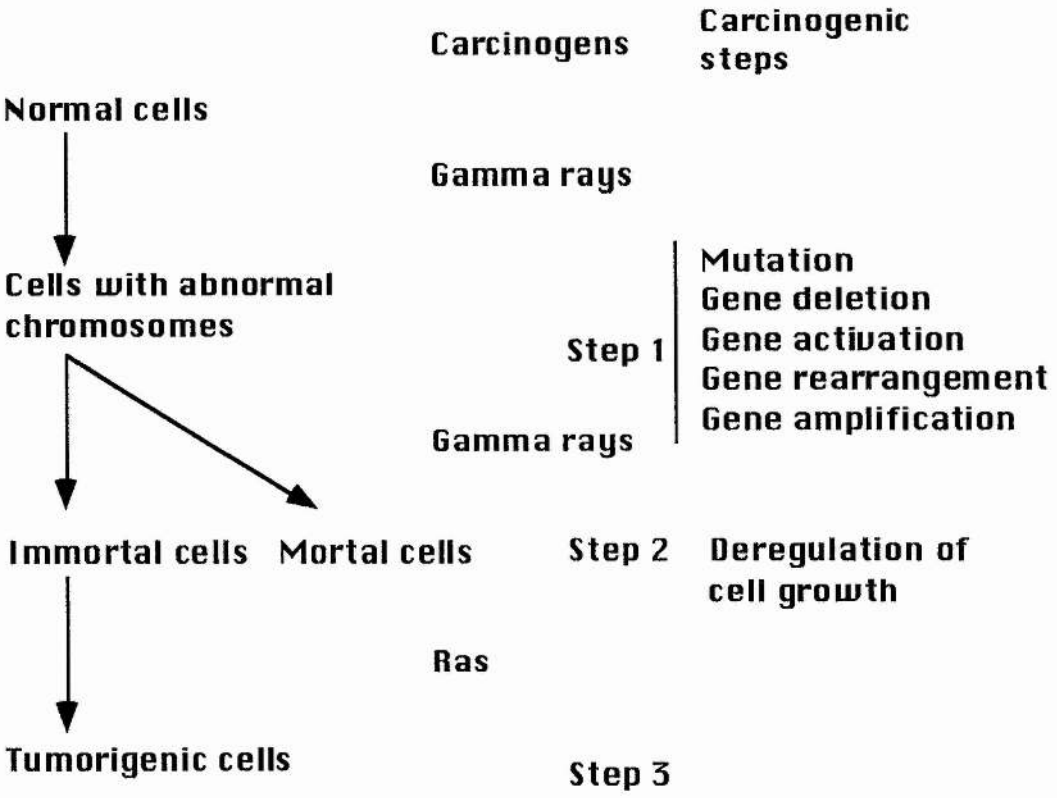


Figure 1.3.2. Multi-step process of neoplastic transformation of human fibroblasts (redrawn from Namba *et al.*, 1988).

capable of directly and irreversibly altering the native molecular structure of the genetic component (DNA) of the cell (Pitot, 1981).

1.3.2. Promotion

Promotion is defined as the process whereby initiated cells are expanded forming nodules, papillomas, or polyps, which can act as an origin for the ultimate development of cancer (Farber, 1984). In contrast to initiation, promotion takes place over a long period of time and is reversible up to the moment of development of the first autonomous tumour cell.

Although the mechanism of tumour promotion is not well understood, there is evidence that the primary target of the tumour promoters is the cell membrane. Recent studies suggest a model for promotion in which the promoting agent produces a differential effect on the initiated cells compared to the unaltered cell, causing a focal proliferation of initiated cells (Archer, 1992).

A promoting agent is one that alters the expression of genetic information of the cell (Pitot, 1981). The diterpene phorbol ester, and 12-O-tetra-decanoylphorbol-13-acetate (TPA), the active component of croton oil, are examples of potent promoting agents. They do not directly interact with the genetic material, and are not in themselves carcinogens. Ionizing radiation has the properties of initiating agents, since it alters the basic structure of DNA. However, it also possess promoting activity and therefore may be considered as complete carcinogen (Pitot, 1981).

1.3.3. Tumour progression

Progression is defined as the stepwise process whereby the occasional expanded initiated cell, the nodule, the polyp, or the papilloma, evolves into a cancer (Farber, 1984). Tumour progression is the least understood process in carcinogenesis. It is not known how much genetic damage is responsible for tumour progression however, there are hints that oncogenes may be involved. Insertional mutagenesis by retroviruses (Varmus, 1982: Nusse, 1986), and point mutations induced in proto-oncogenes by chemical carcinogenesis (Balmain & Pragnell, 1983; Barbacid, 1986; Brown *et al.*, 1986) may on occasions exemplify initial steps in tumorigenesis. There is evidence that mutation of one of the *ras* proto-oncogene family members may account for the appearance of a new and

more aggressive variant of the tumour cell population (Vousden & Marshall, 1984).

1.3.4. *In vitro* neoplastic transformation is a multistep process

The neoplastic transformation of human cells *in vitro* is also a multistage phenomenon by which normal human cells aquire a transformed phenotype (Rhim, 1989; Rhim, 1992). The following three factors appear to be necessary for the malignant transformation of normal cells in culture or *in vivo*: (a) DNA damage, (b) deficient DNA repair during G2 phase of the cell cycle, and (c) continued proliferative stimulus from activation of protooncogenes or loss of tumour suppressor genes (Sanford & Parshad, 1991). None of these factors alone is able to induce neoplastic transformation.

Four major changes have been proposed (Rhim, 1993b) to be involved in neoplastic transformation *in vitro*:

(a) development of morphological transformation

(b) growth in semisolid medium

(c) infinite life span

(d) tumorigenicity

From the studies on neoplastic transformation of cells in culture described above it appears that at least two, possibly more, distinct steps are required for full transformation of normal human epithelial cells *in vitro*.

1.4. Cellular senescence and *in vitro* transformation of human cells

Until recently, one of the biggest obstacles in studying neoplastic transformation of human cells *in vitro* has been the inability to maintain these cells in culture. When normal human cells are kept in culture, after a fixed number of cell doublings, they reach a stage of no proliferation in which the cells become quiescent. This phenomenon is known as cellular "senescence" (Hayflick & Moorhead, 1961) and is observed with all types of human cells.

Since the phenomenon of cellular senescence is a dominant trait of all cultured human cells, it is assumed that all cell lines established in cell transformation experiments *in vitro* arise as a direct consequence of a deliberate carcinogenic insult to normal human cells (Chang, 1986). In support of this assumption was the fact that normal human cells in culture, unlike rodent cells, do not or very rarely undergo spontaneous transformation.

The resistance of normal human cell cultures to spontaneous transformation *in vitro* is in direct contrast to rodent cells, where spontaneous transformation occurs frequently and is accompanied by a change in chromosomal ploidy, expression of other transformation markers, and tumorigenicity (DiPaolo, 1983).

1.5. Criteria for neoplastic transformation *in vitro*

The difficulties in defining neoplastic transformation of human cells is impeded by the lack of unanimous agreement on an end point when the cultured cells become malignant. In previous reports on human cell

transformation studies, several different criteria were used to define transformation. One of the major problems of these *in vitro* studies is that it is impossible to utilize the usual criteria of carcinogenesis used *in vivo* to identify neoplastic cells. Some of the numerous characteristics that are regarded to be indicative of cell transformation *in vitro* are the following:

morphological changes
anchorage-independent growth
extended life span
immortality
reduced serum requirement
enhanced growth rate
increased saturation density
loss of contact inhibition of cell replication
chromosomal aberration
impairment or loss of terminal differentiation
tumorigenicity in susceptible hosts

While these markers have been originally used in defining the transformed phenotype of rodent fibroblasts, it is now evident that many are not applicable for the identification of a transformed human phenotype. As an example, anchorage independent growth (AIG) in rodent cells is acquired as a late marker and is in turn usually correlated with tumorigenicity (Shin *et al.*, 1975). In human cells, in contrast, this marker is expressed early or late and may not be correlated with tumorigenicity (DiPaolo, 1983; Chang, 1986). Indeed there are some human tumour cell lines which are not AIG but, which are clearly tumorigenic in nude mice (Marshall *et al.*, 1977). In this context, AIG by some transformed human cells may reflect not so much transformation *per se* but a growth factor

requirement, a notion that is supported by the observation that normal human fibroblasts can be induced to AIG by high serum or hydrocortisone (Peehl & Stanbridge, 1981).

In those cells which exhibited one or more of these changes but which were not tumorigenic, have been defined as partially or incompletely transformed (Rhim, 1993). Additional steps are therefore necassary for complete transformation. Neoplastic transformation of human cells *in vitro* is defined here as complete transformation, ie the transformed cells produce tumours in athymic nude mice. This is in accordance with the terminology recommended by the Tissue Culture Association, that is to say, the term *in vitro* neoplastic transformation is "the acquisition, by cultured cells, of the property to form neoplasms, benign or malignant, when inoculated into animals" (Schaefer, 1983).

1.6. Assays used for *in vitro* transformation studies

For many years, there have been numerous efforts to develop suitable *in vitro* models for studying the mechanisms of carcinogenesis. Such efforts resulted in succesful development of rodent transformation systems, and relatively little success with human cells. Initially, the development of rodent cell systems for studying *in vitro* the conversion of normal cells to the cells possessing the neoplastic phenotype has allowed a closer examination of the phenomena associated with the neoplastic transformation of rodent cells.

The most commonly used systems at that time were the established lines of mouse embryo fibroblasts, C3H 10T1/2 cell line (Reznikoff *et al.*, 1973) and BALB/3T3 cell line (Kakunaga, 1973), which are sensitive to postconfluence inhibition of cell division (see section 1.7.).

The development of these *in vitro* transformation systems has made it possible to assess the carcinogenic potential of a variety of physical (mainly ionizing radiation), chemical and viral agents. These cell culture systems, free of host-mediated homeostatic influences, afford the opportunity to study, under defined conditions, the cellular and molecular mechanisms involved in radiation-induced transformation.

Advances in the techiques of DNA-mediated gene transfer have led to the development of DNA transfection methods which have been used for identification (Shih *et al.*, 1979; Shih *et al.*, 1981, Hopkins *et al.*, 1981; Krontiris & Cooper, 1981) and molecular cloning (Pulciani *et al.*, 1982; Goldfarb *et al.*, 1982) of dominant transforming genes from neoplastically transformed human cells. This method is based on the fact that the malignant phenotype can be transferred to a mouse fibroblast cell line NIH 3T3 by incorporation of DNA isolated from transformed cells (Shih *et al.*, 1979; Cooper *et al.*, 1980; Perucho *et al.*, 1981).

DNA derived from human cells was transfected using the calcium phosphate transfection procedure (Graham & van der Eb, 1973; Shih *et al.*, 1979) into the mouse fibroblast cell line NIH 3T3, and foci of transformants were scored 14-20 days later (Figure 1.6.1.). Using human, rabbit and rat tumour DNAs in this assay, it was demonstrated that tumour transforming genes are able to act across species barriers, and also tissue barriers. For example, dominant transforming genes of epithelial cells can transform the recipient fibroblasts (Shih *et al.*, 1981).

Blair *et al.* (1982) described a useful system in which the nude mice assay was used together with the conventional focus assay, providing additional sensitivity. The assay detects dominant transforming genes in the DNA of transformed human cells. DNA prepared from human cells are transfected into the mouse cell line NIH 3T3, and upon appearence of

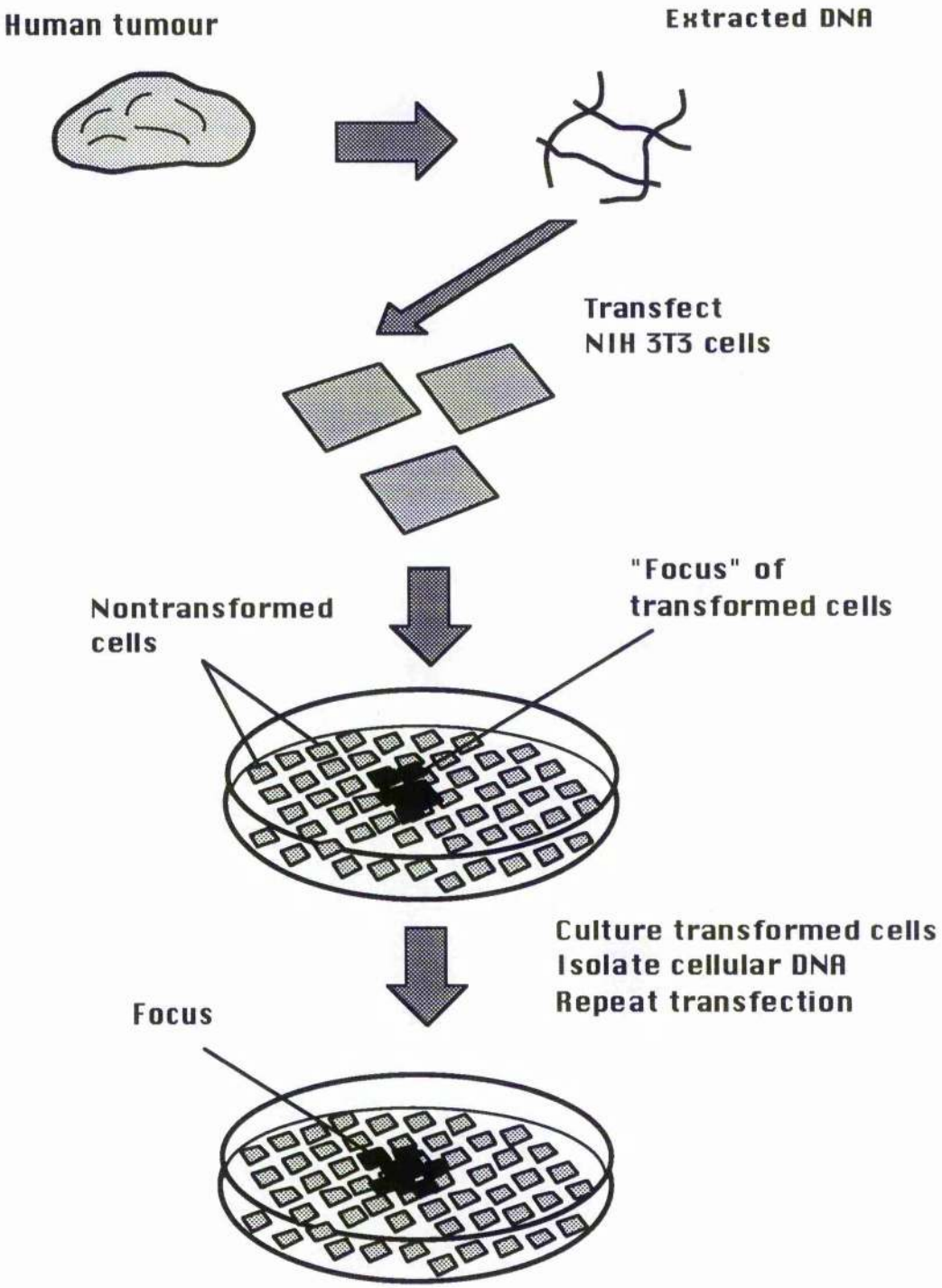


Figure 1.6.1. Standard assay for detecting oncogenes (redrawn from Watson *et al.*, 1992).

transformed foci, cells from these foci were injected into athymic nude mice for tumorigenicity testing.

To prove that the acquired human sequences were responsible for the tumour induction, DNA can be isolated directly from the tumour and screened for the presence and expression of transfected sequences. This model avoids the difficulties associated with monitoring and maintaining tissue culture for many weeks and eliminates the time-consuming microscopic screening of large numbers of tissue culture dishes. However, there is a background of spontaneous foci always present in this assay, and potentially obscures detection of transforming genes.

A modification of this method has been described by Fasano *et al.* (1984), which also relies on the ability of transformed NIH 3T3 cells to form tumours in nude mice, but in addition incorporates technique of cotransfection to heighten sensitivity. The biochemical marker G418 was utilized in cotransfection with human DNA, allowing only G418-resistant colonies to grow. Selection for the G418 marker preselected for cells competent in the incorporation of DNA (Wigler *et al.*, 1979) and removes from the population spontaneously transformed NIH 3T3 cells which have not incorporated DNA.

Another assay using transfection of DNA from transformed human cells into mouse NIH 3T3 cells by radiation fusion has been described (Freyer *et al.*, 1993). Radiation fusion was carried out by irradiating transformed human cells with 1000 Gy of γ-rays and fusing them to NIH 3T3 cells with polyethylene glycol (PEG). Plates containing transformed human/NIH 3T3 fusion cells yielded transformed foci.

1.7. Neoplastic transformation of rodent cells *in vitro*

Most published work concerning *in vitro* neoplastic transformation has been performed on rodent cells. Initially, primary hamster embryo cell cultures were used in studies of X-ray transformation *in vitro* (Borek & Sachs, 1966; Borek & Hall, 1973). The protocol for the assay of neoplastic transformtion in hamster embryo cells is schematically presented in Figure 1.7.1. Briefly, hamster embryos are removed, minced, enzymatically dissociated, and seeded as single cells into culture dishes. They are then treated with radiation and allowed to grow for 8 to 10 days, and the resultant colonies are fixed and stained. Transformed colonies are identifiable by piled up cells and the crisscross pattern at the colony edge.

Subsequently, interest has centered on the use of established mouse embryo fibroblast cell lines which are sensitive to the postconfluence inhibition of cell division (Terzaghi & Little, 1976a; Terzaghi & Little, 1976b; Little, 1977; Miller & Hall, 1978). The two cell lines on which most work has been focused are the mouse fibroblast systems: C3H $10T^1/_2$ (Reznikoff *et al.*, 1973a; 1973b) and the 3T3 system (Todaro & Green, 1963; Kakunaga, 1973).

These two cell lines have several advantages over primary cell cultures. As they are established lines, they have an infinite life span and can be grown in large quantities. Since the cells can be cloned, the same cloned cell population may be used by many research groups. This has significant importance for reproducibility of results among different laboratories (Terzaghi & Little, 1976; Miller & Hall, 1978; Han & Elkind, 1979).

Of the two cell lines, the C3H 10T1/2 cell line is most frequently used, this is because it exibits a lower spontaneous transformation incidence.

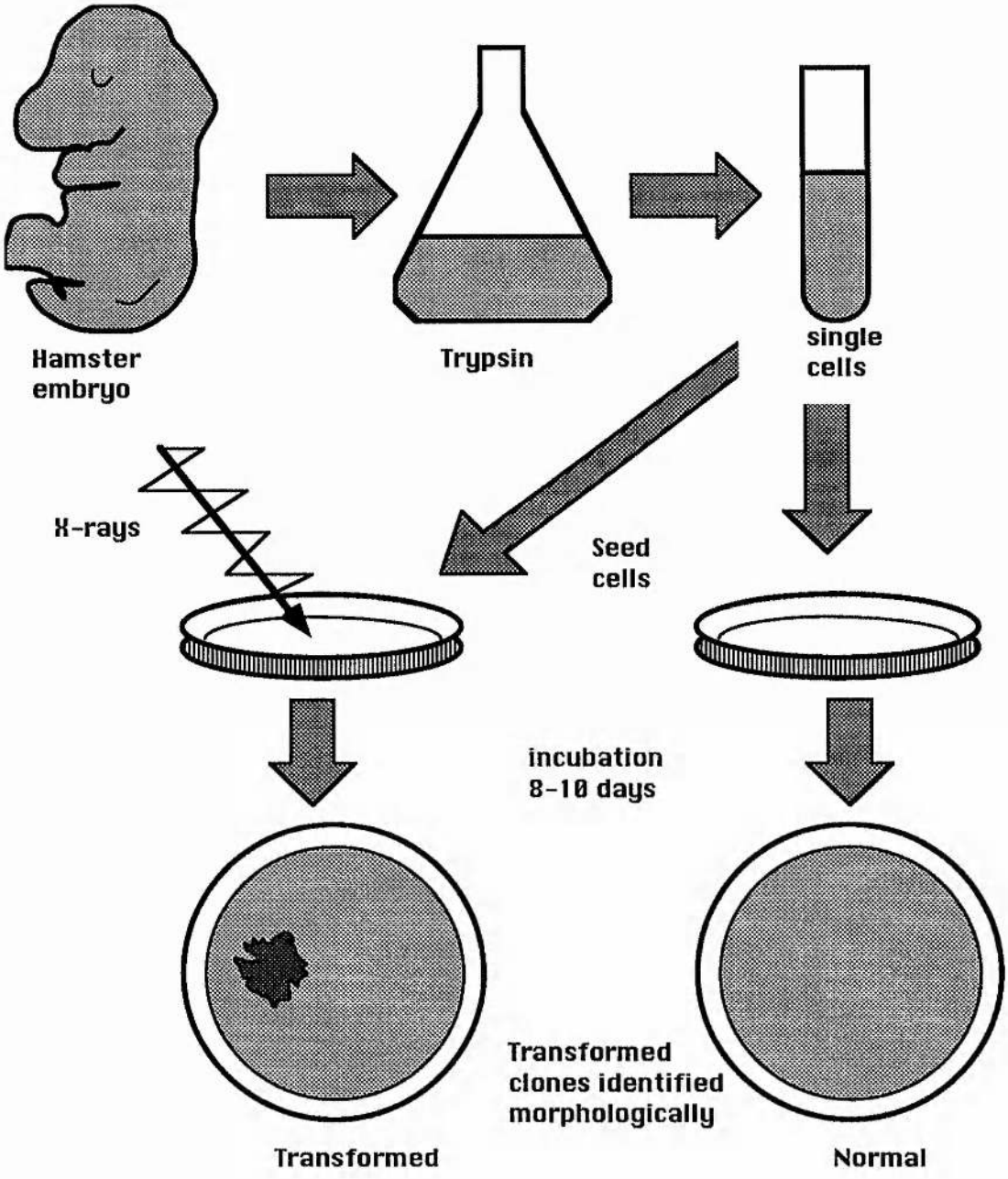


Figure 1.7.1. Protocol for the assay of neoplastic transformation in hamster embryo cells by radiation (redrawn from Hall & Miller, 1981).

The way in which the radiation-induced transformation experiments were carried out with C3H 10T1/2 cells is schematically presented in Figure 1.7.2. Cells are seeded into culture dishes and allowed to attach overnight at 37°C, then are exposed to irradiation, and incubated for 6 weeks, with a weekly change of medium. At the end of this period the cultures are fixed, stained and scored for transformed foci type II and III using the criteria described by Reznikoff *et al.* (1973a). These criteria include piling up, and criss-crossing of cells into opaque multilayers.

Murine fibroblast systems have been extensively used for studies of radiation-induced neoplastic transformation *in vitro*. These include studies on dose-rate effects (Han *et al.*, 1980a; 1980b; Hill *et al.*, 1982), LET dependence (Hei *et al.*, 1988), cell cycle dependence (Cao *et al.*, 1992; Miller *et al.*, 1992), and the effect of fractionated doses (Miller & Hall, 1978; Hill *et al.*, 1985; Miller *et al.*, 1988; Bettega *et al.*, 1992). In addition to radiation studies, these systems were intensively used for studying effects of chemical carcinogens (Reznikoff *et al.*, 1973a; Kakunaga, 1973), and restriction enzymes (Bryant & Riches, 1989)

These studies have demonstrated that the efficiency of radiation-induced transformation depends on the total doses, dose rate, radiation regime, and quality of the radiation.

Studies with low-LET radiation showed that the number of transformants per irradiated cells increases with dose initially, over the range where little cell killing occurs (i.e., the shoulder of the cell-survival curve). At higher doses, as cell killing increases, the observed number of transformed foci decreases (Little, 1977). Studies with charged particles (Hei *et al.*, 1988), neutrons (Hill *et al.*, 1982; Hill *et al.*, 1985; Miller *et al.*, 1988), and α-particles (Hieber *et al.*, 1987; Bettega *et al.*, 1992) demonstrated that

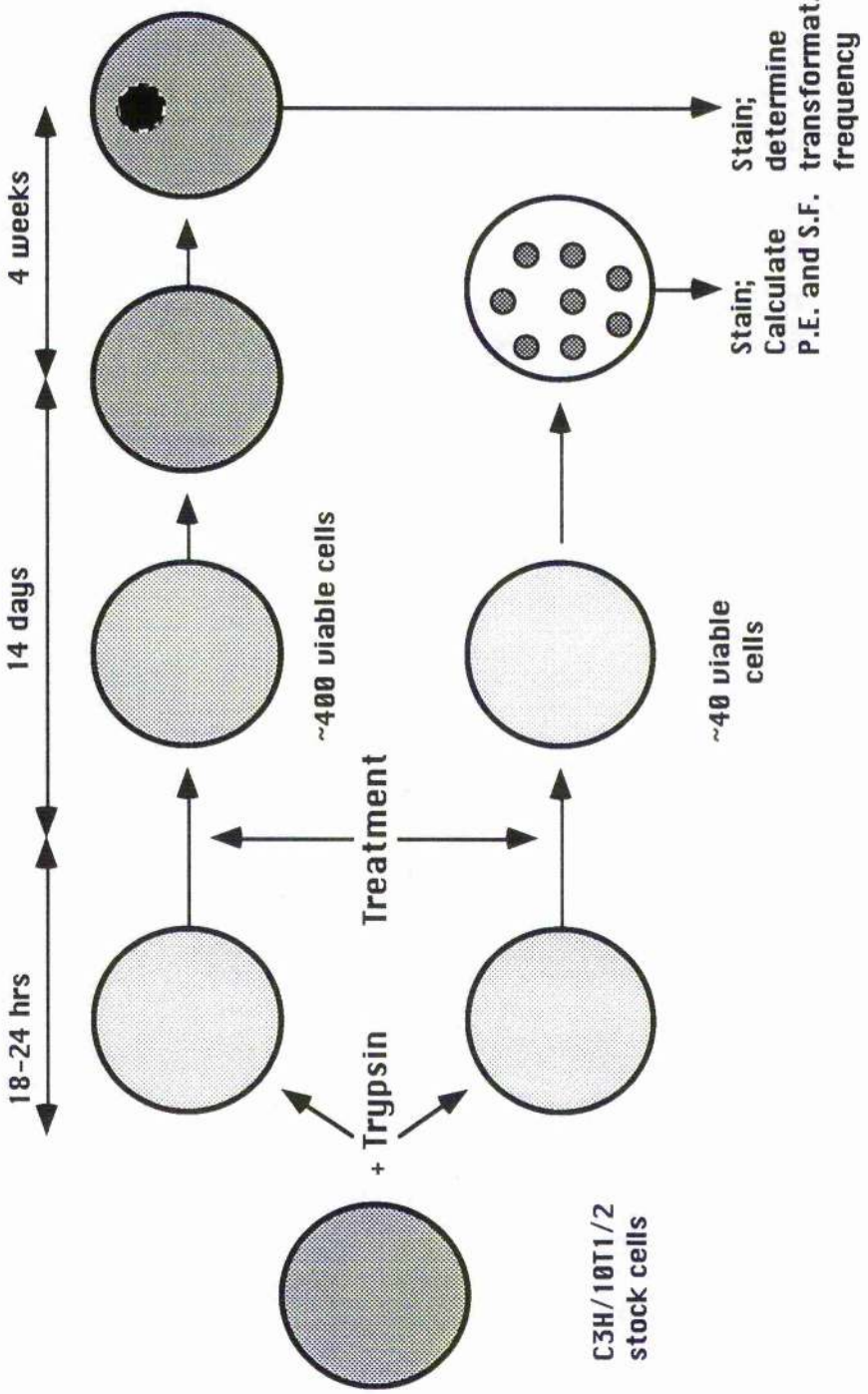


Figure 1.7.2. Protocol for experiments with 10T1/2 cells (redrawn from Hall & Miller, 1981)

high LET radiation is far more cytotoxic and oncogenic than low LET radiation.

Data obtained with ^{60}Co γ-rays indicated that reduction of the dose rate resulted in a marked reduction in transformation frequency (Han *et al.*, 1980a; 1980b). In contrast, Hill *et al.* (1982) demonstrated that reduction of the dose rate of neutrons resulted in an increase in transformation frequency (so-called "inverse dose-rate effect" for neutrons). Enhancement of transformation frequency was obtained by fractionated doses of X-rays (Miller & Hall, 1978), neutrons (Hill *et al.*, 1985; Miller *et al.*, 1988), and of α-particles (Bettega *et al.*, 1992).

Radiation-induced neoplastic transformation has also been shown to be cell-cycle dependent. Studies with mouse C3H 10T1/2 cells (Cao *et al.*, 1992; Miller *et al.*, 1992) demonstrated that cells at late G_2- and M-phase of cell cycle had a maximal sensitivity to neoplastic transformation by γ-radiation.

The data obtained with hamster embryo cells demonstrate that the dose-response curves for the induction of transformation by ionizing radiation has a characteristic shape (Borek & Hall, 1973). It consists of three portions: an ascending limb, a plateau and a descending limb (Figure 1.7.3.). The transformation frequency increases as the dose is increased up to a plateau, which corresponds to the doses of between 1.5 Gy and 3 Gy. A further increase in the dose results in a marked decrease in the transformation incidence. Similar dose-response relationships have been obtained using human and animal *in vivo* data. Studies of the dose-response relationship in radiation-induced cancers *in vivo* indicated the tendency for the dose-response curve to reach a saturation point and decline at high levels (Mole 1958; Bond *et al.*, 1960; Upton, 1961; Gray,

1965). This phenomenon is particularly conspicuous in the induction of thyroid thumours by ^{131}I in rat (Lindsay *et al.*, 1957; Potter *et al.*, 1960).

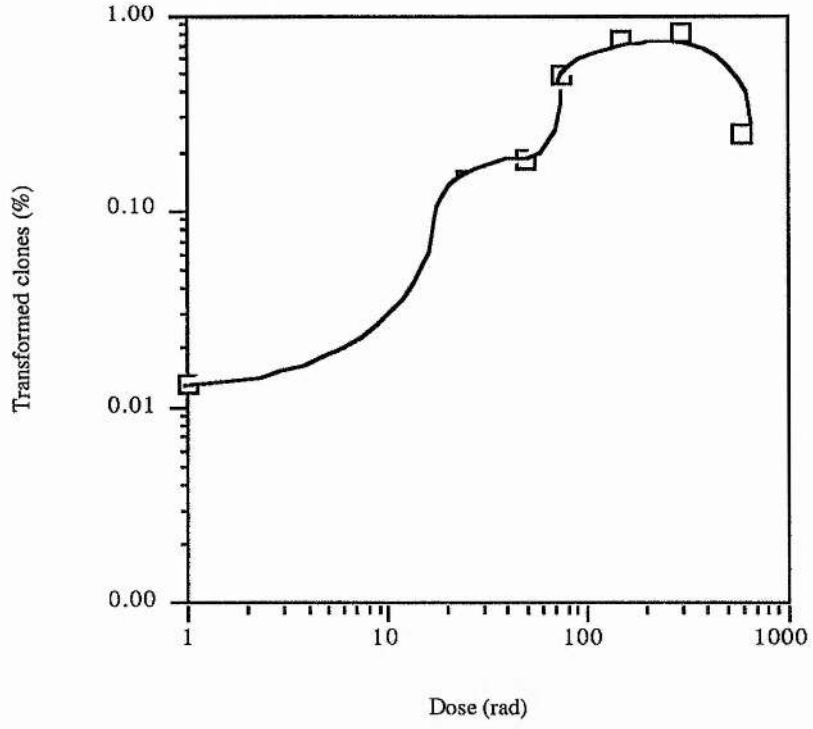


Figure 1.7.3. Incidence of hamster embryo cell transformation following exposure in vitro to X-irradiation (redrawn from Borek and Hall, 1973).

Dose-response data obtained in *in vitro* and *in vivo* studies suggest that those irradiated cells that are destined to become transformed cells are more susceptible to killing by the accumulation of additional damage at higher doses. In other words, many of the potentially transformed cells never have the chance to form colonies or tumours because they die of reproductive death due to the extra damage produced by the additional damage received at higher doses (Gray, 1965; Borek & Hall, 1973).

Similarly, the transformation frequency of mouse embryo C3H 10T1/2 cells following X-irradiation, based on the number of transformants per surviving cell, was found to increase exponentially up to a certain

dose, after which any further increase in the dose resulted in little change to the transformation frequency (Terzaghi & Little, 1976).

It is interesting that splitting the dose into two fractions enhances the transformation frequencies at low doses. In contrast at high doses fractionation results in less effect than the same total dose in a single exposure (Miller & Hall, 1978). Borek & Hall (1974) also found that split doses of X-rays induced higher transformation frequences in fresh explants of hamster embryos. Since radiation transformation involves sublethal events, it is not surprising that splitting the dose leads to less killing but enhanced transformation incidence (Borek & Hall, 1974).

1.8. Neoplastic transformation of human cells *in vitro*

The studies utilizing murine fibroblast systems have largely contributed to the present knowledge of neoplastic transformation *in vitro*. It must be admitted, however, that these systems suffer from the shortcomings of being rodent and fibroblastic in origin, raising doubts about their relevance to human epithelial carcinogenesis (Barrett, 1991). Extrapolation of data from experimental carcinogenesis to the human situation would be more logical if human cells rather than animal cells are utilized (DiPaolo, 1983). Therefore, the use of human cells in studying neoplastic transformation is essential for understanding the cellular and molecular mechanisms underlying human carcinogenesis.

Unlike rodent cells, human cells do not or rarely undergo spontaneous transformation, and have generally proven resistant to *in vitro* neoplastic transformation by carcinogenic agents (Di Paolo, 1983; Chang, 1986; Rhim, 1989). The basis for differences in transformation sensitivity is not yet understood. Several explanations, however, may be

speculated such as difference in relative genetic stability and natural life span.

Transformation studies of human cells have predominantly been undertaken with fibroblasts, which are relatively easy to culture. The most important approaches for the study of the transformation of human cells *in vitro* include exposure of the cells to physical agents (e.g., γ-, X-, and ultraviolet irradiation), chemical carcinogens and viruses.

1.8.1. Effect of chemicals on human cells

Despite many attempts, neoplastic transformation of human cells in culture by chemical means has not been readily achieved. The first report of neoplastic transformation of normal human cells by a chemical carcinogen was published in 1978 (Kakunaga, 1978). Human diploid skin fibroblasts (KD) were exposed to 4-nitroquinoline-1-oxide and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and alteration of cell growth patterns, unlimited life span and tumorigenicity in athymic nude mice were observed. However, it was subsequently found that the normal fibroblastic cell line (KD) and transformed HuT cell lines were derived from different individuals (McCormick *et al.*, 1988). It was further demonstrated that the HuT series of "carcinogen-transformed" human fibroblast cell lines were derived from the human fibrosarcoma cells 8387 (McCormick *et al.*, 1988). In 1983, neoplastic transformation of human diploid fibroblasts *in vitro* by N-acetoxy-2-acetylaminofluorene was reported (Zimmerman & Little, 1983b).

There have also been a few reports describing transformation of human epithelial cells by chemicals. Chemical transformation of human

pancreas cell explants (Parsa *et al.*, 1981a; 1981b; Parsa *et al.*, 1984), and human foreskin epithelial cells (Milo *et al.*, 1981) are reported.

More recently, neoplastic conversion of human epidermal keratinocytes immortalized by a hybrid of adenovirus 12 and simian virus 40 (Ad 12-SV40) (Rhim *et al.*, 1986) and human urothelial cells (Reznikoff *et al.*, 1988) by chemical agents have been reported.

1.8.2. Effect of biological agents on human cells

The most successful approach to *in vitro* neoplastic transformation of human cells has been by treatment of cells by viral agents, either as infectious particles or viral DNA. The list of successful reports on the neoplastic transformation of normal human cells by biological agents has been growing. With few exceptions, neoplastic transformation has been achieved by transfection of two agents: one for immortalization and another for conversion of immortalized cells. Among the most frequently used agents for immortalization of human cells are viral agents such as the Simian virus 40 (SV40) (for a review see Chang, 1986), and Human Papilloma virus (HPV). In a few cases a hybrid containing the adenovirus 12 and simian virus 40 transforming genes (Ad 12-SV40) (Schell *et al.*, 1966) has been used (Rhim *et al.*, 1981; 1985; 1986).

1.8.2.1. SV40 immortalization of human cells *in vitro*

The most widely used agent for immortalizing human cells is SV40, first isolated in 1960 (Sweet & Hillman, 1960). SV40 is a member of the papova family which are small, nonenveloped DNA tumour viruses. The virus contain a covalently closed circular duplex DNA molecule of 5243

base pairs (bp) which can be functionally divided into early and late regions transcribed in opposite directions (Figure 1.8.2.1.). In "permissive" cells, the early region is transcribed throughout the lytic cycle, and differential splicing produces two mRNAs that encode the large T and small t proteins. The late region encodes the viral capsid proteins (VP1-3) and is transcribed only after the onset of viral DNA replication. During the late phase coat proteins are made and progeny virions are assembled. Release of mature virus particles results in lysis and cell death.

Monkey cells are "permissive" for SV40 infection, i.e. they support full expression of the viral genome resulting in the release of mature viral particles. Mouse cells, however, are "non-permissive" and human cells "semi-permissive" for SV40, which are both unable to support viral replication with any efficiency. SV40-infected murine cells produce no progeny virus, but express some of the viral genes and generate new properties in the cells hence they become "transformed". SV40 infection of human cells however, can lead to either the development of a "transformed" phenotype or of lytic infection with the release of virus particles.

The transformation of mammalian cells by SV40 is known to require the expression of only the early region of the viral genome, which encodes two proteins, large T-antigen (94 kDa) and the small t-antigen (17 kDa). While the large T-antigen is necessary for initiation of transformation and maintenence of the transformed phenotype, small t-antigen, although not essential for transformation, may have a qualitative effect on this event.

Various types of human cells have been "transformed" by SV40 or large T-antigen (for a review see Chang, 1986; Manfredi & Prives, 1994). Two basic approaches have been used in transforming human cells in culture by SV40: viral infection and SV40 DNA transfection. Human cells

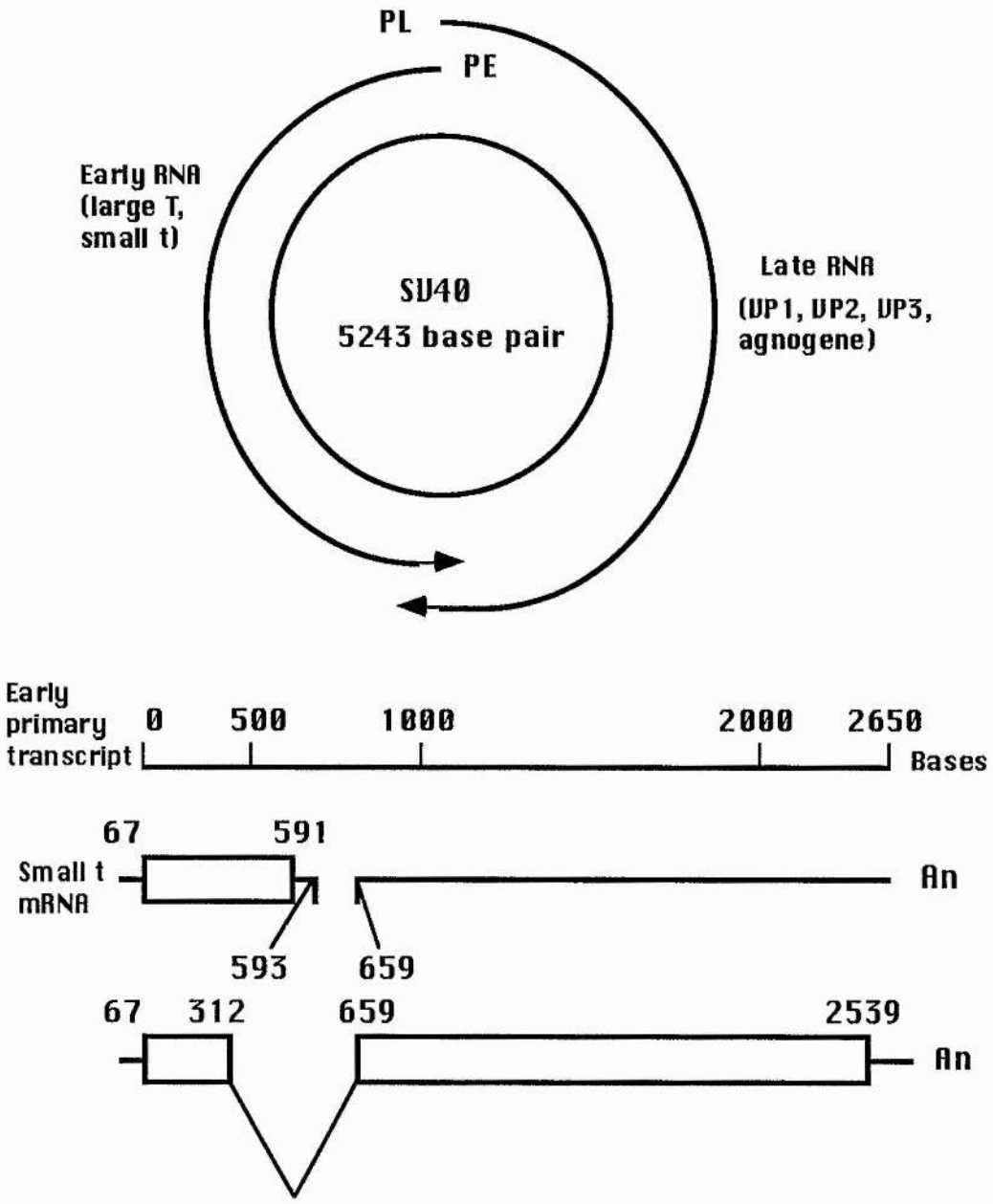


Figure 1.8.2.1. SV40 gene expression. Two primary transcripts are expressed from opposite strands that are further processed to produce early and late viral mRNA. These transcripts initiate from the early (PE) and late (PL) SV40 promoters (redrawn from Benchimol, 1992).

infected with SV40 form foci of altered cells after 2-7 weeks post-infection. SV40 infection of the cells can be easily demonstrated by indirect immunofluorescence staining of nuclear large T-antigen. Transformed

cells have an extended life span. However, the cells eventually reach a stage where there is little or no net proliferation and cells continue to cycle, but, die at such a high rate that most of them are lost. This is known as the phenomenon of "crisis" (Girardi *et al.*, 1965) resulting in the escape of variant SV40-transformed human cells with infinite life span.

Another technique often used to transform human cells by SV40 is calcium-phosphate-mediated transfection.(Graham & van der Eb; 1973). This technique is particularly useful when human cells are transformed with SV40 mutants, e.g., SV ori$^-$, which lacks a functional viral origin of replication through a deletion of six nucleotides at the *Bgl* I site (Gluzman *et al.*, 1980). It was found that SV ori$^-$ has an enhanced frequency of transformation of human cells in comparison to the normal SV40 (Small *et al.*, 1982). This can be explained by the fact that the transfected DNA was unable to replicate and consequently unable to cause cell death through the lytic cycle.

The mechanism of SV40 transformation is not yet fully understood. It has been demonstrated that the SV40 proteins bind to the p53 gene and retinoblastoma gene products (Rb), both negative regulators of cell growth (Lane & Crawford, 1979; Linzer & Levine, 1979; DeCaprio *et al.*, 1988). Both gene products act as checkpoint "monitors", which stop the progression of cells through the cell cycle if the conditions are not satisfactory (Hartwell & Weinert, 1989; Murray, 1992, Hartwell, 1992; Levine *et al.*, 1994). By binding to the p53 and Rb gene products, viral proteins neutralize and inactivate them thereby inactivating the checkpoint controls. It was found that p53 protein when bound to T-antigen can no longer bind to the responsive DNA element and fail to act as a transcription factor (Mietz *et al.*, 1992)

Until recently little was known about the site of SV40 integration in the human genome. Evidence from early studies of hybrids between SV40-

transformed human cells and mouse cells, suggested that the integration of active SV40 in human cells was site-specific on chromosome 7 (Croce *et al.*, 1973) However, subsequent work has demonstrated that SV40 integrates nonspecifically at other sites, including at least two different locations on chromosome 7 (Campo *et al.*, 1979), chromosome 8 (Kucherlapati *et al.*, 1978), and chromosome 17 (Croce, 1977). Experiments with SV40-immortalized human fibroblasts also suggested that no specific integration sites in the cellular genome exist, which is a prerequisite for the immortalization process (Hara & Kaji, 1987).

1.8.2.2. Neoplastic conversion of human cells by biological agents

Human cells immortalized by SV40 or other agents were neoplastically transformed by various viral or cellular oncogenes (Table 1.8.2.1.).

However, it is interesting to note that oncogenes alone did not convert normal human cells into either immortal or neoplastically transformed cells (Rhim *et al.*, 1985; O'Brien *et al.*, 1986; DiPaolo *et al.*, 1989) Thus, the immortalization of human cells appears to be an indispensable step for the neoplastic transformation.

1.8.3. Effect of ionizing radiation on human cells

Despite the fact that the carcinogenic action of ionizing radiation in humans has been well documented from epidemiological studies (BEIR, 1972; Rossi & Kellerer, 1974; UNSCEAR, 1977), immortalization and neoplastic transformation of human cells in culture by the treatment with ionizing radiation has proven extremely difficult to achieve.

Table 1.6.1. The *in vitro* neoplastic transformation of human epithelial cells by biological agents.

Cells	Immortalization agent	Transforming agent	Reference
Bronchial	Ad12-SV40	v-H-ras	Amstad *et al.*, 1988
Bronchial	Ad12-SV40	Ki-MSV	Reddel *et al.*, 1988
Bronchial	-	v-H-ras	Yoakum *et al.*, 1985
Cervical	HPV-16	v-H-ras	DiPaolo *et al.*, 1989
Keratinocytes	Ad12-SV40	Ki-MSV	Rhim *et al.*, 1985
	Ad12-SV40	c-H-*ras*	Rhim, 1989
	Ad12-SV40	retrovirus	Rhim *et al.*, 1988
	Ad12-SV40	v-*fos*	Lee *et al.*, 1993
	pSV3 neo	Ki-MSV	Gantt *et al.*, 1987
	HPV	Ki-MSV	Durst *et al.*, 1989
	spontaneous	c-H-*ras*	Boukamp *et al.*, 1990
Kidney	Nickel	v-H-*ras*	Haugen *et al.*, 1990
Mammary	Benzo(*a*)pyrene	retrovirus	Clark *et al.*, 1988
	-	SV40 Tag	Berthon *et al.*, 1992
Urinary tract	SV40	c-H-*ras*	Christian *et al.*, 1988

Only one case of immortalization of normal human cells by a physical agents has been reported (Namba *et al.*,1985). Normal fibroblasts (KMS-6) derived from a human embryo were immortalized producing the KMST-6 line by repeated treatment with ^{60}Co γ-rays. However, despite a large number of experiments only one immortalized cell line was established. Little *et al.* (1991) reported, however, failure to induce immortalization of human diploid fibroblasts by single or multiple exposures to X-irradiation in a total of 46 separate experiments. This

finding suggests that immortalization is a rare and rate limiting event in neoplastic transformation of human diploid cells.

Most studies involving the neoplastic transformation of human cells have been carried out with fibroblasts, which are relatively easy to culture. Borek (1980) was the first to document the *in vitro* neoplastic transformation of human cells by ionizing radiation. Human skin fibroblasts (KD) were exposed to X-rays (4 Gy) and growth in soft agar, and tumorigenicity in nude mice were observed. However, cells derived from the tumours developed in nude mice following this treatment have not been characterised.

There is only one report of neoplastic transformation following extensive passaging (at 547th passage and 2,800 days after initiation of culture) of human fibroblasts that were previously immortalized with repeated ^{60}Co γ-rays (Mihara *et al.*, 1992). Therefore, there are no reproducible human cell systems for radiation-induced *in vitro* neoplastic transformation (DiPaolo, 1983; Chang, 1986; McCormick & Maher, 1988; Rhim, 1992).

1.8.4. Neoplastic transformation of human epithelial cells by ionizing radiation

Since epithelial cells are the cells of origin of most human cancer, an epithelial cell culture system would be of significant importance for the study of the cellular changes and the molecular mechanisms which occur during malignant transformation. Until recently, the difficulties in culturing human epithelial cells made it difficult to induce and detect neoplastic transformation of human cells *in vitro*. In the last decade, as a result of improved methods for culturing human epithelial cells, and the

application of new tools from molecular biology, the field of human epithelial cell transformation has seen many successes.

A neoplastic transformation of human cells has been achieved in a stepwise fashion in a similar way to those described previously with oncogenes and viruses (section 1.6.2.). Normal human cells were immortalized, mostly by a DNA virus or a construct of viral genome, and then converted to tumorigenic cells by subsequent treatment with ionizing radiation. Thraves *et al.* (1990) showed that nontumorigenic human epidermal keratinocytes (RHEK-1), previously immortalized by a hybrid Ad12-SV40, can be malignantly transformed by fractionated exposure to X-ray irradiation. The transformed cells formed colonies in soft agar and showed morphological alterations. These altered cells induced tumours when transplanted into nude mice.

Using energetic heavy ion beams, Yang *et al.* (1991) succeeded in transforming human mammary epithelial cells and human epidermal keratinocytes to various stages of transformation. Human mammary epithelial cells (H185B5) and human epidermal keratinocytes (RHEK) previously immortalized with benzo(a)pyrene (Stampfer, 1985) and pSV3-neo (Gantt, 1987), respectively, have an infinite life span and are nontumorigenic in nude mice. After repetitive exposure to high-LET heavy particles (2.2 Gy of iron particles) human mammary cells formed anchorage independent colonies. However, in spite of considerable efforts, it was not possible to achieve this with only a single irradiation.

Following irradiation human epidermal keratinocytes showed morphological alterations which include focus formation with piling up of cells in dishes. These altered cells are also able to grow in soft agar but, do not form tumours when injected into athymic nude mice. However, when

given a further exposure to radiation, they became tumorigenic when injected into nude mice (Yang *et al.*, 1991).

Yang *et al.* (1994) have also reported the developmement of an *in vitro* human epithelial cell system for neoplastic transformation of human epithelial cells by ionizing radiation (Figure 1.8.4.1.). Immortal nontumorigenic human mammary epithelial cells (184B5) were irradiated with heavy ion beam, and focus assay was used to detect morphological transformation of growth variants. These transformed cells, however, did not produce tumours in athymic nude mice, and therefore can be regarded as partially transformed.

Hei *et al.* (1994) recently demonstrated that immortalized human cells in culture can be malignantly transformed by a single dose of α-particles. Neoplastic transformation of papilloma virus-immortalized human bronchial epithelial cells (BEP2D) was achieved by a single 30 cGy dose of α-particles, and subsequent subculture for a period of 3 months. Transformed cells produced progressively growing tumours upon transplantation into nude mice.

A further report of radiation-induced neoplastic transformation of human epithelial cells was published by Martin *et al.* (1993). Working with immortalized human uroepithelial cells (HUC) and immortalized human epidermal keratinocytes (RHEK), neoplastic transformation was achieved by a low single dose of ^{4}He α-particles. Following irradiation, cells were passaged for six weeks and injected into athymic nude mice. Tumours were obtained and tumour cell lines established.

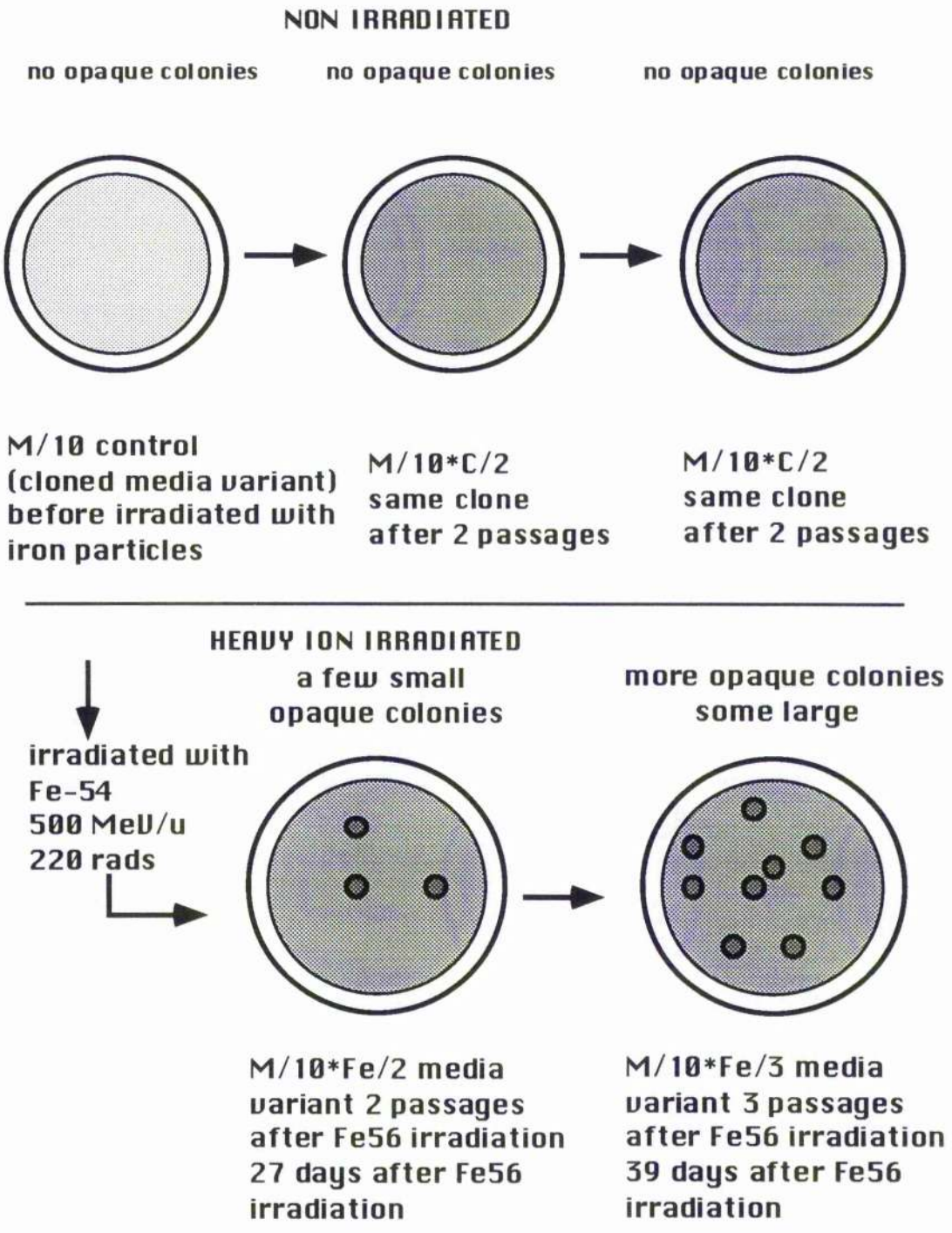


Figure 1.8.4.1. Transformation of human mammary epithelial cells 18B5 F5-1 (clone M/10) by 600 MeV iron particles. The transformed foci were found in irradiated cells and persisted for several passages in culture (redrawn from Yang and Craise, 1994).

1.9. Radiation-induced thyroid cancer

Studies in man and animals have demonstrated that the thyroid gland is highly susceptible to the induction of neoplastic lesions by ionizing radiation (UNSCEAR, 1964). A study of children treated with X-rays for alleged thymic enlargement (Simpson *et al.*, 1955), and scalp ringworm (Albert and Omran, 1968; Modan *et al.*, 1977) showed a significant increase in the incidence of thyroid cancinomas and benign thyroid neoplasms. In addition, in the years since the Chernobyl accident, there has been a substantial increase in the incidence of thyroid carcinomas in children in Southern Belarus and in Northern Ukraine (Williams *et al.*, 1993). It was suggested that radioactive isotopes of iodine released from the damaged reactor are the most likely cause of the post-Chernobyl increase in childhood thyroid cancer found in these regions.

Lemoine *et al.* (1988) reported that DNA from 60% of radiation-induced rat thyroid tumours transfected into NIH3T3 cells scored positive in the nude mouse tumorigenesis assay. Southern blot analysis showed that in eight out of nine cases, transforming activity was due to the K-*ras* oncogene activation (Lemoine *et al.*, 1988). *Ras* mutations have been shown to occur both in "spontaneous" and radiation-associated human thyroid tumours (Yoshida *et al.*, 1988; Lemoine *et al.*, 1989b; Wright *et al.*, 1991b).

From pathological evidence, neoplasia of human thyroid follicular cells appears to progress along two distinct pathways (Wynford-Thomas, 1993). These are "follicular" and "papillary" pathways, both arising from the follicular cells, and culminating in the agressive anaplastic carcinoma (Figure 1.9.1.). "Follicular" neoplasia retain the characteristic structural

element of the normal gland. "Papillary" neoplasia differ both in morphology and biological behaviour (Wynford-Thomas, 1993).

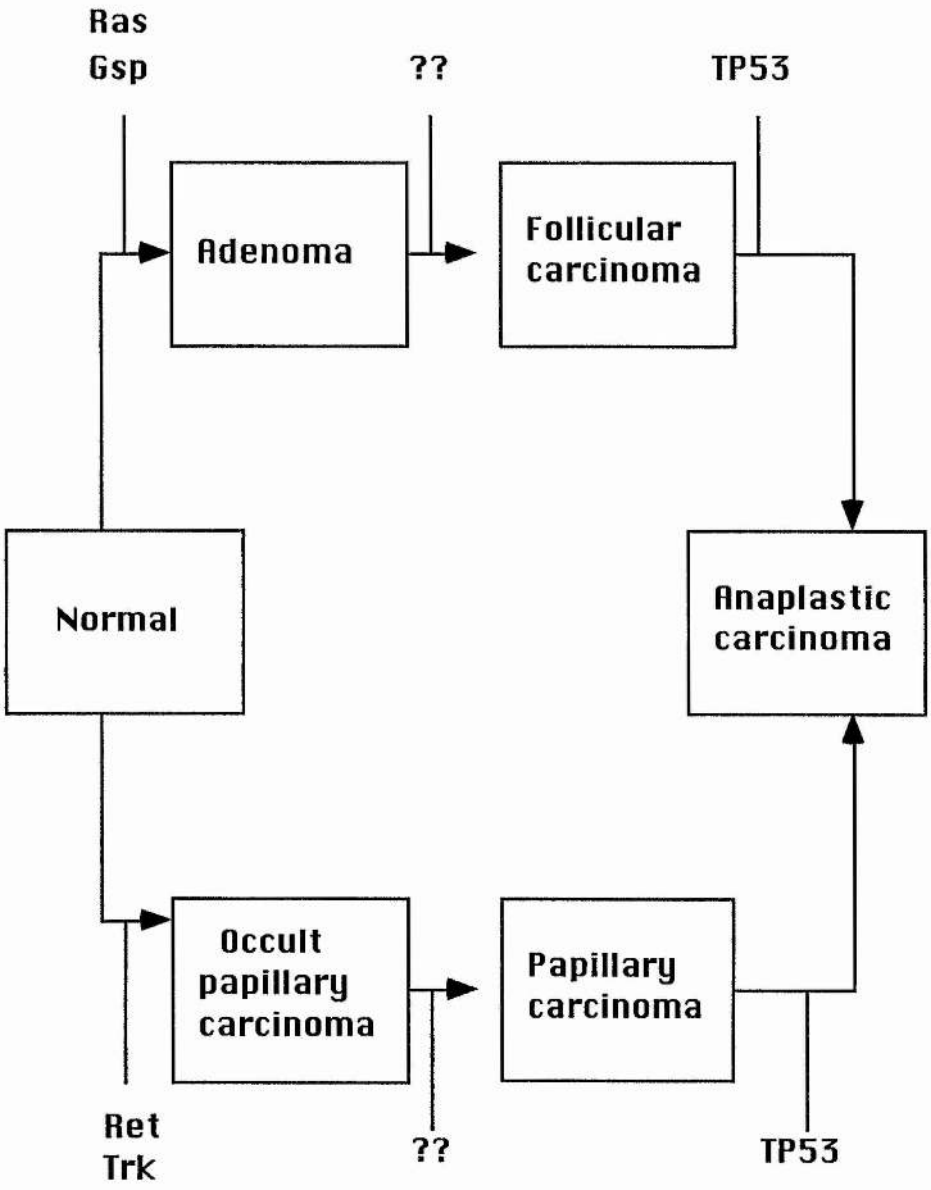


Figure 1.9.1. Multi-stage tumorigenesis in human thyroid follicular cells (redrawn from Wynford-Thomas, 1993).

Numerous studies have been undertaken to investigate whether this contrast in biological behaviour might be associated with different patterns of oncogene activation. Lemoine *et al.* (1988) found a marked difference in the frequency of activated *ras* oncogenes detectable by transfection assays in papillary cancers (20%) compared with follicular cancers (80%). This finding suggested a connection between this pattern of oncogene activation and the marked difference in biological behaviour.

In addition, frequent rarrangements of the *ret* oncogene have been observed in papillary tumours but not in follicular tumours (Ishizaka *et al.*, 1989; Grieco *et al.*, 1990; Pierotti *et al.*, 1992; Jhiang *et al.*, 1992; Williams & Williams, 1995). Ito *et al.* (1993) reported inducibility of rearranged *ret* oncogene in a thyroid derived cell line following *in vitro* X-irradiation.

Studies of human thyroid tumours suggested that p53 mutation plays a role in the progression of differentiated thyroid carcinomas to undifferentiated ones (Ito *et al.*, 1992). However, other studies failed to demonstrate p53 mutations in human thyroid tumours (Wright *et al.*, 1991).

1.10. Aims of thesis

The cellular and molecular mechanisms of radiation-induced neoplastic transformation of human epithelial cells are poorly understood. This is mainly due to the difficulties in obtaining a suitable *in vitro* model of human cell neoplastic transformation. An optimal model would provide an easy induction and detection of neoplastic transformation of human epithelial cells by single dose of radiation. The main aims of this study were the following:

◊ Induction of neoplastic transformation of human thyroid epithelial cell line by γ- and α-irradiation.

◊ Investigate the effect of different types of ionizing radiation (γ, α) on transformation incidence.

◊ Investigate the effect of different irradiation regimes (single and multiple exposure) and different post-irradiation treatment (number of passages) on transformation frequency.

◊ Examine the existence of molecular and cellular changes involved in the neoplastic process.

◊ Characterize the tumour cell lines produced.

Chapter 2

MATERIALS AND METHODS

2.1. Cells and media

2.1.1. The HTori3 cell line

The human thyroid epithelial cell line, designated HTori3, was used for the transformation studies. This cell line was established from human primary thyroid follicular epithelial cells following transfection with a plasmid containing a replication origin defective SV40 genome (5.3 kb SV40 genome with a six base pair deletion that eliminates the BgII site at the origin of replication) (Lemoine, 1989). The resultant immortal cell line retains normal epithelial morphology and expresses specific features of thyroid epithelial function (iodide-trapping and thyroglobulin secretion), at early passages forms anchorage-independent colonies at low frequency but, is not capable of forming tumours in athymic nude mice. This line is aneuploid in nature and has 44-76 chromosomes per metaphase, with no clear mode. Southern blot analysis confirms the integration of a single copy of SV40 DNA.

The HTori3 cells were supplied by professor D. Wynford-Thomas (University of Wales College of Medicine, Cardiff, United Kingdom). The cells were grown in 80 cm^2 flasks [Nunc] at 37°C. The medium used was a

mixture of equal volumes of Dulbecco's MEM [Gibco BRL] and Ham's F12 medium [Gibco] supplemented with 7% (v/v) foetal calf serum (FCS) [Globepharm], penicillin 100 units/ml [Sigma], streptomycin 100 μg/ml [Sigma], and L-glutamine 2 mM [Flow Laboratories].

2.1.1.1. Cell passage

Cells were routinely subcultured at subconfluence at a 1:20 split ratio. All the medium was removed and the flasks were washed twice with 4 ml of a solution of 0.05% (w/v) trypsin [Difco] and 0.2% EDTA [Sigma] in phosphate-buffered saline (PBS). Flasks were then left for 5 minutes in a 37°C incubator. A phase-contrast microscope [Olympus] was used to check whether the cell sheet is completely detached from the flask bottom. Cells were then resuspended in medium, pipetted up and down several times to ensure the separation of individual cells in suspension, and 1/20 plated into new flasks. After passaging, the cultures were gassed with 5% carbon dioxide (CO_2) to reach an optimum pH. Caps were screwed air tight and flasks were kept in a 37°C dry incubator. A complete medium change was carried out every 2-3 days.

2.1.2. The T24 cell line

The T24 human bladder carcinoma cell line was used as a positive control for H-*ras* mutation screening at the codon 12 valine allele (Taparowsky *et al.*, 1982; Capon *et al.*, 1983a). The T24 cells were supplied by Dr J. Masters (Institute of Urology, London, United Kingdom). Cells were cultured in Rosewell Park Memorial Institute tissue culture

medium 1640 (RPMI 1640) [ICN Flow] supplemented with 5% FCS [Globepharm], penicillin [Sigma] 100 units/ml, streptomycin [Sigma] 100 µg/ml, and 2 mM L-glutamine [Flow Laboratories]. The cells were passaged with 0.01% trypsin [Difco] with 0.2% EDTA [Sigma] in PBS at 37°C for 10 minutes.

2.1.3. The SW480 cell line

The SW480 human colon adenocarcinoma cell line, homozygous for the mutant K-*ras* codon 12, was used as a positive control for screening the tumour cell lines for a mutant K-*ras* at the codon 12 valine allele (Capon *et al.*, 1983b). The cells were supplied by Dr C. Paraskeva (Medical School in Bristol, United Kingdom). Cells were maintained in Dulbecco's MEM medium [Gibco BRL] supplemented with 7% FCS [Globepharm], penicillin [Sigma] 100 units/ml, streptomycin [Sigma] 100 µg/ml, and 2 mM L-glutamine [Flow Laboratories]. Cells were removed from the flasks using 0.1% trypsin [Difco] with 1% EDTA [Sigma] in PBS at 37°C for 4 minutes.

2.1.4. The HOS cell line

The human osteocarcinoma cell line (HOS) was used as a positive control for mutant P53 (Romano *et al.*, 1989). The HOS cells were provided by Dr. B. Vojtesek (Department of Biochemistry, University of Dundee, United Kingdom). Cells were grown in Dulbecco's MEM medium [Gibco BRL] supplemented with 10% FCS [Globepharm], penicillin [Sigma] 100 units/ml, streptomycin [Sigma] 100 µg/ml, and 2

mM L-glutamine [Flow Laboratories]. Cells were detached from the flasks using 0.05% trypsin [Difco] and 0.1% EDTA [Sigma] in Hank's balanced solution (pH 7.2) at 37°C for 20 minutes. Medium was changed every 2-3 days.

2.2. Maintenance of the cell lines

2.2.1. Freezing

Stocks of original HTori3 cells were kept frozen in liquid nitrogen and used when required. When a cell line has been produced, seed stock was stored frozen in liquid nitrogen. The cell culture was grown to a late log phase and the resulting high density monolayers were trypsinized as describe above (section 2.1.1.). Approximatelly 1-1.5 x 10^7 cells were resuspended in culture medium (equal mixture of Dulbecco's MEM and Ham's F12 medium) containing 20% FCS. As a preservative 10% dimethyl sulphoxide (DMSO) [BDH] was added dropwise and equilibrated by gently swirling. The cell suspension was aliquoted (1.5 ml) into 2 ml cryotubes [Nunc]. The tubes were placed initially in the gas phase of a liquid nitrogen freezer which has slow cooling rate for at least 24 hours. The tubes were then transfered to the liquid phase (-196°C) for longer storage.

2.2.2. Thawing

The tubes were retrieved from liquid nitrogen and quickly thawed by immersion in the water bath at 37°C. When thawed, the tubes were

swabbed with 70% alcohol and the content of the tubes was transfered to culture flasks. Culture medium (10 ml) was then added slowly dropwise to the cell suspension to avoid osmotic damage. The flasks were gassed with 5% CO_2 and placed into the incubator at 37°C for 6-9 hours to allow the cells to attach. Medium was then changed to further dilute the DMSO, thereby reducing toxicity.

2.2.3. *In situ* detection of mycoplasma in cell cultures

Cell cultures were periodically examined for mycoplasma contaminants using Hoechst stain [Flow Laboratories]. This method takes advantage of the DNA-intercalating dye Hoechst 33258 which excites at 360 nm and emits at 490 - 500 nm. Contaminated cultures are detected by the bright, punctuate cytoplasmic staining of the mycoplasma DNA.
The cells were grown on slides under the condition described in section 2.5.2. When the cells reached 50-80% confluency, they were fixed by adding Carnoy's fixative (1 part glacial acetic acid to 3 parts absolute methanol) and incubated at room temperature for 5 minutes. The fixation step was repeated by adding Carnoy's fixative for an additional 10 minutes, and then the slides were air dryed for 30 minutes.

The working dilution of Hoechst stain, 0.05 µg/ml in the Hanks Balanced Solution (HBS) was added for 30 minutes at room temperature. Slides were washed 3 times with distilled water at room temperature allowing 1 minute soaking time per wash, then were air dryed. The cells were then examined using a fluorescent microscope [Carl Zeiss] under oil immersion. No mycoplasma contamination was detected in our cultures.

2.3. Transformation studies

2.3.1. Gamma-irradiation procedure

The experimental design is shown in Figure 2.3.1.1. Subconfluent cells attached to flasks were exposed to single or multiple doses of γ-radiation from a ^{137}Cs IBL 437C γ-irradiator [CIS UK Bio International] at a dose rate of 4.6 Gy per minute. After irradiation cells were plated into flasks, re-fed with fresh medium every 2-3 days, allowed to reach subconfluence, and subcultured 1:10 once between each fractioned dose to maintain continuous proliferation. When the cells reached subconfluence they were irradiated again. Sets of control unirradiated cells were handled in exactly the same way. Following the final irradiation, cells were passaged 6 times (4-6 weeks), after which they were injected into nude mice for tumorigenicity tests. Each flask was maintained independently and passaged without pooling cells and expanded to 3 flasks per group before transplantation.

2.3.2. Alpha-irradiation procedure

Experimental protocol followed for experiments with α-particles is shown in Figure 2.3.1.1. Cells were irradiated with α-particles ^{238}Pu α-particle source at the Medical Research Council Radiobiological Unit in Chilton (Goodhead *et al.*, 1991). Irradiations were carried out by Drs. A. Riches and P. Bryant in collaboration with staff at MRC RBU. The design is shown schematically in Figure 2.3.2.1. The source had half life 86 years and activity of ~1.2×10^9 Bq. The energy spectrum of the α-particles emitted was 5.50 and 5.46 MeV.

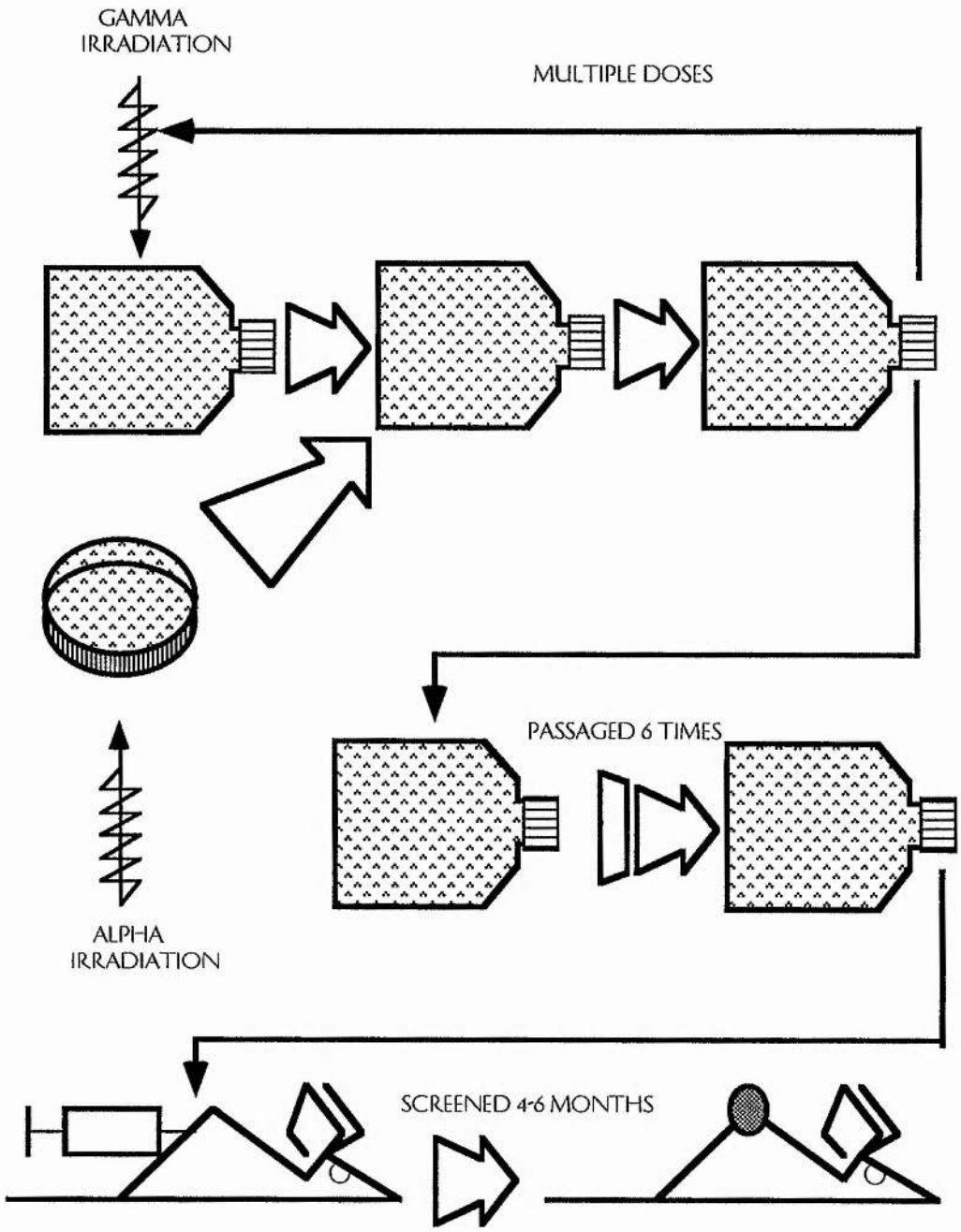


Figure 2.3.1.1. Experimental protocol used for in vitro cell transformation of HTori3 cells by γ- and α- irradiation.

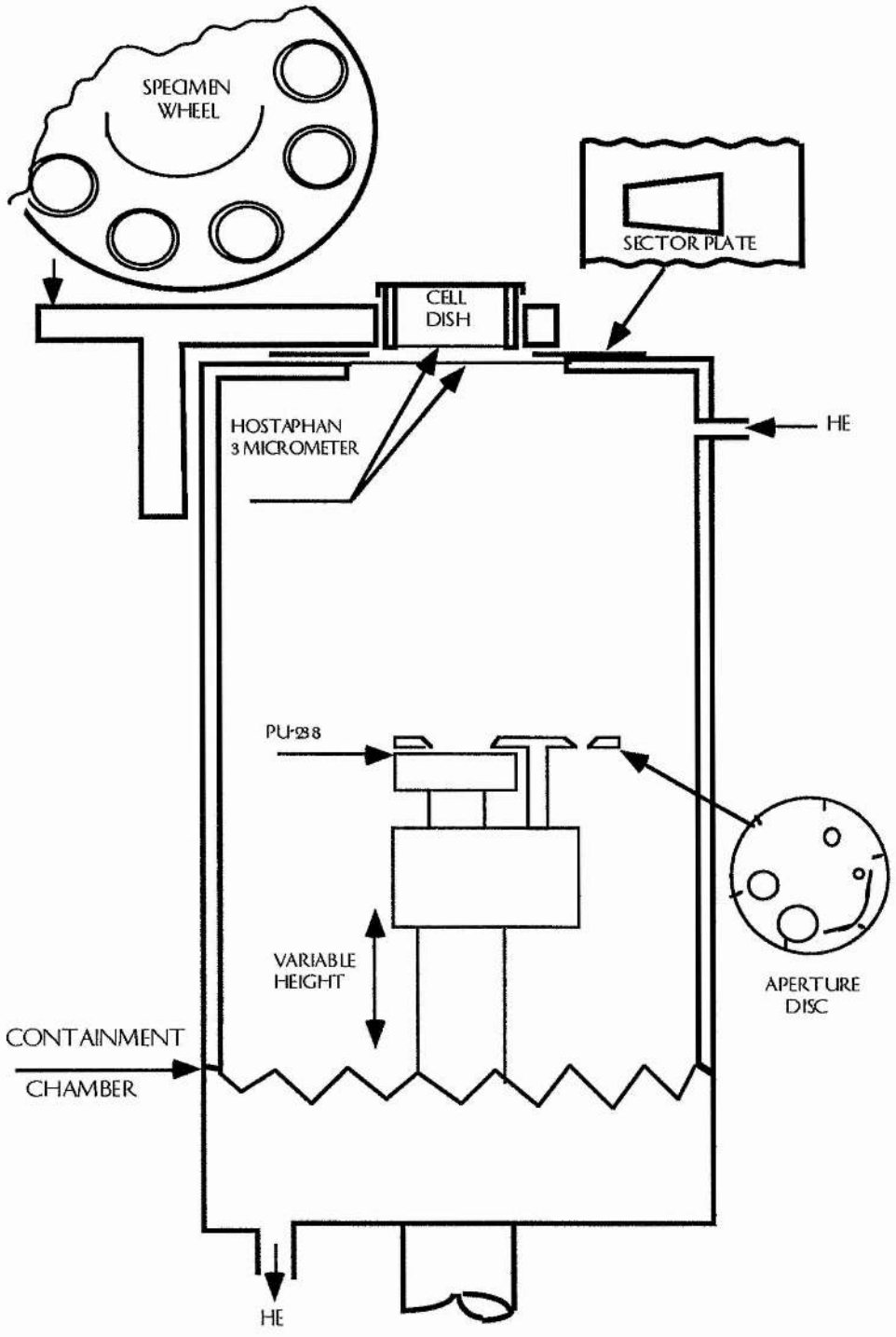


Figure 2.3.2.1. Schematic diagram of the main components of the α- particle source chamber and sample wheel (redrawn from Goodhead *et al.*,1991).

Due to the limited penetration of the α-particles, cells were irradiated in specially designed dishes consisting of a glass ring of 5 cm diameter and a hostaphan bottom of 3 μm thickness. No significant difference was observed in plating efficiency or growth rate with cells grown in these dishes as compared to those cultured in the conventional tissue culture flasks. 2.5 x 10^5 cells were plated 24 hours prior to irradiation to ensure that the cells were in exponential growth during the time of exposure.

Irradiation was performed at room temperature. Immediately following irradiation the central portion of hostaphan bottom was cut out by a sharpened cylindrical punch in order to select only those cells which had received a full dose of radiation. The cells were removed from the bottom by routine trypsinization and plated into flasks. For the higher dose experiment, 2 or 3 dishes were trypsinized and pooled together. Cells of all experimental groups were passaged 6 times before injection into nude mice.

2.3.3. Tumorigenicity assay

To test tumorigenicity of treated cells and derived lines from tumours, the cells were injected into congenitally athymic nude mice (nude mice), strain MF1 nu/nu. The original stock of mice was purchased from OLAC Ltd, but once a breeding stock was established animals were taken for experimental purposes from this stock. The animals were kept and bred in the University of St. Andrews Animal Handling Facility in a 12 hour light/dark illumination cycle. An ambient temperature were kept constant at 22.3°C (72°F) and the air in the rooms

were filtered. Mice were fed with RM1(E) pellets [Special Diet Service] and allowed sterile water *ad libitum*.

Cells to be tested in the tumorigenicity assay were harvested using the trypsin/EDTA technique (see section 2.1.1.), resuspended in medium, transfered into sterile 30 ml universal tubes [Sterilin] and spun down at 1,000 rpm in a Chilspin [MSE Scientific Instruments] for 10 minutes. Supernatant was discarded and the pellet was dispersed in 10 ml of sterile saline. The suspension was spun down again at 1,000 rpm for 10 minutes, supernatant discarded and the pellet resuspended in the minimum amount of the remaininig liquid. The cell number was counted using a Improved Neubauer Hemocytometer [Weber Scientific Instruments].

The cell suspension was sucked into a 1 ml syringe fitted with a 21 gauge needle. Prior to injection, the dorsal region of the nude mice were swabbed with 70% (v/v) ethanol and 2-3 $x10^6$ cells suspended in 0.1 ml sterile saline were inoculated subcutaneously.

Nude mice (4-6 weeks old) of both sexes were used for injection. The animals were monitored for tumour formation for up to 6 months. The growth of tumours was measured weekly by comparing the volume of the tumour with graded size steel ball bearings (Riches & Thomas, 1970).

When the tumour reached 1 cm in diameter, or after a 6 months experimental period, animals were sacrificed, and tumours were dissected in order to establish tumour cell lines and also for histopathological examination. For histological examination the tumour sections from each animal were fixed in formaldehyde/NaCl solution (1:9 by vol.), embedded in paraffin, sectioned and stained in hematoxylin and eosin (H&E) as described previously (Luna, 1968).

2.3.4. Statistical analysis

Data obtained in transformation experiments were analysed using G-test (Sokal & Rohlf, 1981). The computation of G was performed by the following formula:

G = 2 [sum of $f \ln f$ of frequencies in each cell of the table - sum of $f \ln f$ of column sums of frequencies - sum of $f \ln f$ of row sums of frequencies + $f \ln f$ of total number of items in the table]

Where f represents observed frequency of the particular group

2.4. Establishment of the tumour cell lines

The technique applied for the establishment of the tumour cell lines is schematically presented in Figure 2.4.1. When the tumours had reached suitable size, mice were sacrificed by cervical vertebrae dislocation. Tumours were dissected and trimmed from connective tissue in a Petri dish using sterile technique. A slice of tumour tissue was cut out from the central part of tumour and further chopped into small pieces of approximately 1 mm^3 using a sterile scalpel. The fragments were placed into a 25 cm^2 culture flask with a small amount of F12/Dulbecco's medium supplemented with 1% FCS. Preliminary studies showed that low serum content restricts fibroblast proliferation. Cultures were incubated for 5-6 days at 37°C. This allowed tumour fragments to attach firmly to the bottom of the flask before being disturbed. After this period the cultures were fed every 2-3 days until

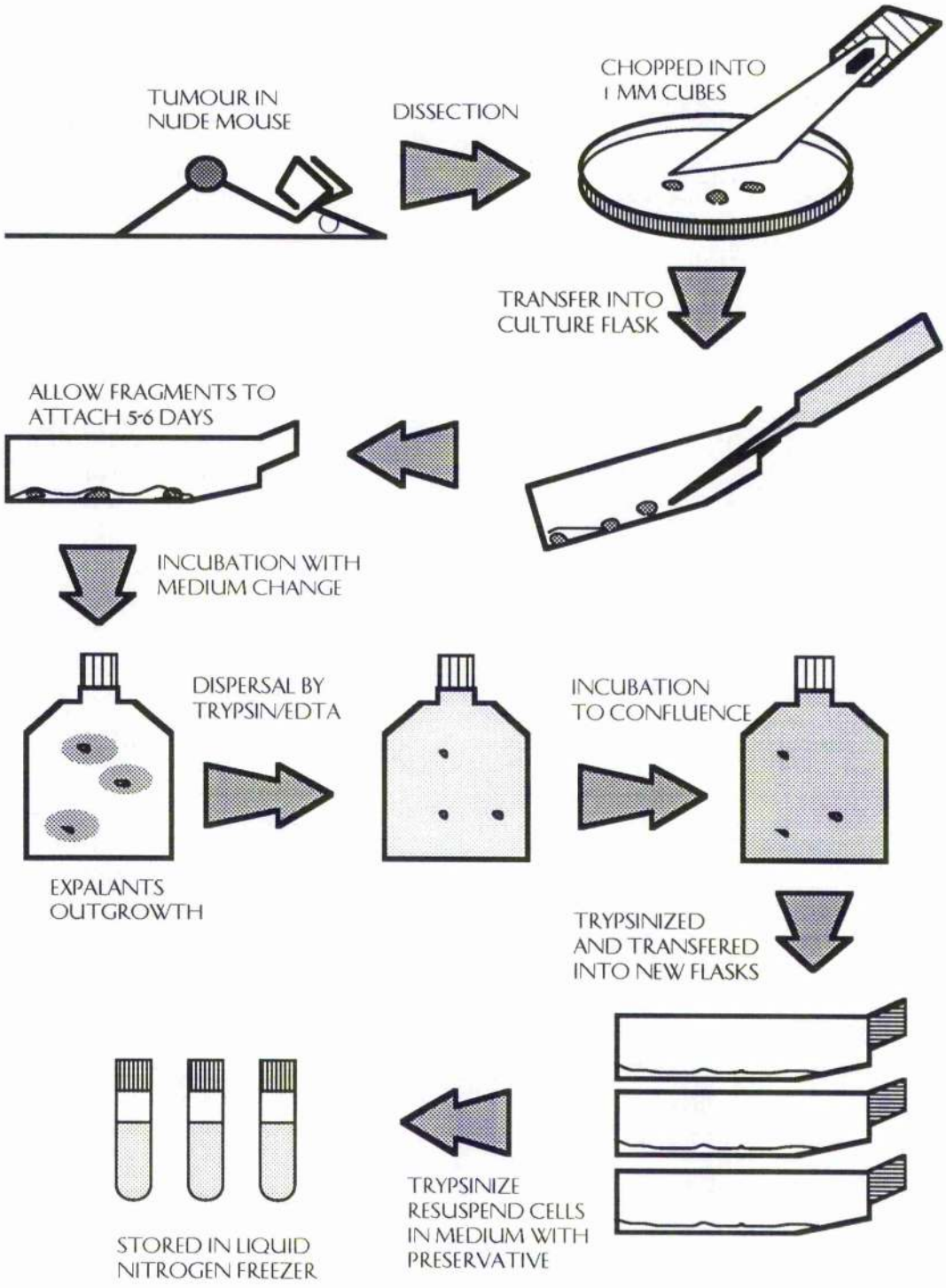


Figure 2.4.1. The technique used for the establishment of cell lines from the primary tumours induced by irradiation.

reaching confluence. When confluent, the cultures were subcultured with trypsin/EDTA mixture (see section 2.1.1.) at a split ratio of 1:3 into 3 new flasks in order to obtain sufficient cell numbers for freezing. Before freezing, cell identity was performed by karyotype analysis, cytokeratin analysis, and DNA fingerprinting.

2.5. Identification of the tumour cell lines

2.5.1. Chromosome analysis

In order to distinguish between human and murine cells in the tumour explants, analysis of chromosomes at metaphase was performed. For this purpose glass slides were specially prepared. Glass slides (72 x 26 mm) [BDH] were first immersed in 1 M hydrochloric acid (HCl) overnight and rinsed with water. Following overnight immersion in 100% ethanol, the slides were air dried, placed in a clean box and cooled to -20°C before use.

Cells were grown in 80 cm^2 flasks and treated for 3-4 hours with 0.04 µg/ml of colcemid (stock solution 4 µl/ml) [Sigma] at 37°C. Cells were then harvested with trypsin and spun in a Chilspin at 1,000 rpm for 10 minutes. Following the removal of supernatant, the cell pellet was resuspended in the remaining liquid and exposed to hypotonic 0.75 M KCl solution (2 ml) for 3 minutes at 37°C.

The cells were fixed by washing the preparations 3 times with cold Carnoy's fixative (methanol : glacial acetic acid = 3 : 1). Following the final washing of the cells and discarding the supernatant, the cells were resuspended in a small volume of fresh fixative.

The cells were dropped onto an ice-cold wet microscope slide using a Pasteur pipette, and immediately warmed by hand. Slides were rapidly dried by heat, and directly stained with 3% (v/v) Giemsa [BDH] in water for approximately 20 minutes. After rinsing in water, the slides were air dried and analysed using a light microscope [Leitz Wetzlar] under oil immersion.

2.5.2. Cytokeratin staining

Detection of cytokeratin (a marker for human epithelial origin) in the tumour cell line was used for establishing the human origin of the cells.

Cells (50 µl of approximately 1 x 10^6/ml single cell suspension) were plated on sterile slides and left overnight to attach in 5% CO_2 fully humidified atmosphere at 37°C. Slides were rinsed with PBS, fixed in cold acetone [BDH] for 20 minutes and rehydrated in 1% sheep serum in PBS (SSPBS) [SAPU] for 30 minutes. Incubation with primary antibody was then carried out at 37°C for 1 hour in a humidified atmosphere. A murine anti-cytokeratin antibody MNF 116 [DAKO] diluted 1 : 25 in 1% SSPBS was used as the primary antibody.

In order to remove the unbound primary antibody, the slides were washed 3 times with PBS. The secondary antibody, a sheep anti-mouse polyclonal antibody S081-201 conjugated with Horderadish peroxidase (HRP) [SAPU] was then added to the slides followed by incubation 37°C for 1 hour. The secondary antibody was then washed off with PBS as described above prior to developing with DAB.

After washing with PBS, the development was performed with 3',3-diaminobenzidine (DAB) [Sigma]. DAB was used at a concentration of 1 mg/ml in 50 mM Tris pH 7.6 with 0.3% (w/v) nickel sulphate (to enhance a black colouration). DAB was activated with 0.06% hydrogen peroxide (H_2O_2) [BDH]. The slides were incubated at room temperature for approximately 5 minutes in the dark before washing and observation.

2.5.3. SV40 T antigen staining

In order to identify the presence of SV-40 T antigen in the established tumour cell lines, immunocytochemical staining was performed. Cells were plated on glass slides and incubated overnight to attach. The slides were fixed with 1 : 1 methanol : acetone for 2 minutes and washed with PBS.

A murine monoclonal anti-T antigen antibody PAb 405 (gift from Prof. David Lane, Department of Biochemistry, University of Dundee) diluted 1 : 500 in PBS was added and incubated for 1 hour at room temperature. The slides were washed 3 times in PBS and then incubated for 1 hour at room temperature with 5% FCS. This was to prevent non-specific binding.

As the secondary antibody a rabbit anti-mouse IgG HRP conjugated polyclonal antibody P161 [DAKO] diluted 1:100 in 1% (w/v) bovine serum albumine (BSA) in PBS (BSAPBS) was used. Incubation was performed at 37°C for 1 hour. Following washing with PBS, the slides were developed with DAB (see section 2.5.2.), washed and photographed.

2.5.4. Immunochemical analysis of p53 protein

The analysis was performed in order to ascertain the presence or absence of mutant p53 proteins in the radiation-transformed cell lines. The procedure can be divided into the following steps:

Cell lysis and protein extraction
Immunoprecipitation with primary monoclonal antibodies
SDS-Polyacrylamide Gel Electophoresis (SDS-PAGE)
Elecrophoretic transfer onto nitrocellulose membrane
Antibody staining of the membrane and visualisation

2.5.4.1. Cell lysis and protein extraction

Confluent cultures (approximately 1×10^7) were harvested, washed 3 times with PBS, and spun at 1,000 rpm for 10 minutes in a Chilspin. After the final washing and pelleting, the cells were either lysed or stored at -70°C. For cell lysis, the NET lysis buffer was used. The NET lysis buffer was prepared as follows:

50 mM NaCl
50 mM Tris [BRL], pH 8.0
5 mM EDTA [Sigma]
1% NP-40 [BDH]
350 µg/ml phenylmethylsulfonyl fluoride (PMSF) [BRL]

NET lysis buffer (1 ml).was added to the cell pellet, vortexed briefly in order to disrupt the pellet and then left 30 minutes on ice. The cell extract was centrifuged at 10,000 rpm (13,000 xg) for 30 minutes at 4°C in a

high speed centrifuge J2-MC [Beckman] and the pellet discarded. The protein extracts were preabsorbed with Protein G Sepharose [Sigma] by adding 40 µl of Protein.G beads suspended in NET buffer to 600 µl aliquots of the protein extracts. This suspension was incubated on rotating wheel 45 minutes at 4°C, and spun at 13,000 rpm for 2 minutes in a minifuge.

2.5.4.2. Immunoprecipitation with primary monoclonal antibodies

200 µl of supernatant from each of samples was transferred into two new tubes containing previously added 1 µl of ascites containing the primary specific antibody, either DO-1, PAb 240 (obtained from Prof. D. Lane). DO-1 antibody reacts with both wild type and mutant type p53, and PAb 240 recognizes only mutant but not wild type p53 (Gannon *et al.*, 1990; Levine 1990; Vojtesek *et al.*, 1992).

In separate tubes, anti-retinoblastoma mutant protein antibody IF8 ("irrelevant antibody") were added to the lysate in order to check for non-specific binding of the second anti-p53 antibody CM1. Following overnight incubation at 4°C, Protein G beads were added and incubation was continued for a further 45 minutes.

The beads (immune complexes) were then washed 4 times with lysis buffer. The lysate and wash buffers were removed by aspiration.

2.5.4.3. SDS-PAGE electrophoresis

After the final wash was completely removed, the beads were resuspended in 40 µl of sample buffer. The buffer consisted of:

4% SDS [BDH]

120 mM Tris [BDH], pH 6.8

20% glycerol [Sigma]

0.002% bromphenol blue [Sigma]

10% mercaptoethanol [BDH]

The samples were heated in boiling water for 10 minutes, spun at 13,000 rpm for 8 minutes in a minifuge and the supernatant either loaded onto the gel or stored at -20°C. Electrophoresis was carried out on a 10% SDS-PAGE. Volumes of the reagents used to cast the gel are given in Table 2.6.3.1.

Table 2.5.4.1. Volumes of reagents used to cast SDS-PAGE gels. [a]Stock solution was prepared by dissolving 29 g of acrylamide [BRL] and 1 g of N,N'-methylenebisacrylamide [BRL] in 100 ml of water. [b]pH of Tris solution was 8.8 and 6.8 for resolving and stacking gel, respectively. [c]Solution was prepared freshly by dissolving 1 g of Ammonium persulfate [BRL] in 10 ml of water.

	Resolving gel		Stacking gel	
Reagent	Concentration	Volume	Concentration	Volume
SDS	10%	0.5 ml	10%	0.1 ml
Acrylamide solution[a]	30%	16.67 ml	30%	1.3 ml
Tris[b]	1.5 M	12.5 ml	0.5 M	2.5 ml
Distilled water		20.33 ml		6.1 ml
APS[c]	10%	400 µl	10%	200 µl
TEMED		40 µl		20 µl

Detailed preparation of the acrylamide solution for the separating and stacking gels are described by Harlow & Lane (1988).

The gel was run on a BRL electrophoresis apparatus, initially at 125 V, and after the dye front had moved into the separating gel, the voltage increased to 200 V for approximately 3 hours. The running buffer (adjusted to pH 8.3) consisted of:

0.025 M Tris
0.24 M glycine [Sigma]
0.1% SDS
distilled water to volume

Prestained SDS-PAGE Standard Solution [Sigma] and unstained Dalton Mark VII-L [Sigma] molecular weight markers were run in parallel to the samples.

2.5.4.4. Electrophoretic transfer onto nitrocellulose membrane

Transfer of proteins from the gel onto the nitrocellulose membrane was achieved using a semi-dry method (Harlow & Lane, 1988) on an LKB 2117 Multiphor II electrophoresis unit. The transfer buffer consisted of:

0.025 M Tris
0.192 M glycine
20% methanol
0.0375% SDS
distilled to volume

The transfer of proteins onto nylon backed nitrocellulose Hybond-C Extra [Amersham] was performed at 0.8 mA per cm^2 of membrane over a period of 1.5 hours.

2.5.4.5. Antibody staining and visualisation

To block non-specific binding sites, the nitrocellulose membrane was incubated at room temperature with 3% BSA [Sigma] diluted in PBS for 2 hours. Following two washes in PBS, the second non-specific anti-p53 antibody CM1, diluted 1:500 in 3% BSA/PBS solution was added and the membrane incubated at 37°C for 1 hour.

The membrane was then washed 4 times in PBS, and incubated with HRP conjugated swine anti-rabbit immunoglobulins [DAKO] diluted 1:500 in 3% BSA/PBS at 37°C for 1 hour. The membrane was then twice washed with PBS prior to developing with DAB (see section 2.5.2.) and photographing.

2.5.5. DNA fingerprinting

The technique of DNA fingerprinting was performed in order to confirm the identity of the cell lines derived from primary tumours. The method can be essentialy split into 4 procedures:

DNA isolation and digestion
Gel electrophoresis and Southern blot
Cloning and radiolabeling of RNA probe
Hybridization and visualization

2.5.5.1. DNA isolation and digestion

Genomic DNA was isolated by phenol/chloroform extraction of proteinase K treated cells followed by restriction enzyme digestion

a) Cell lysis

The cells were harvested with trypsin/EDTA technique (see section 2.1.1.), centrifuged, and washed twice in ice-cold PBS. After the second wash the cellularity was determined by a Coulter counter (see section 2.6.1.1.). The cell pellet was then resuspended in lysis buffer evenly (1 ml of lysis buffer per 1.2 - 2.0 x 10^7 cells) and 1 ml aliquots were transfered into 1.5 ml Eppendorf™ tubes [Scotlab]. The lysis buffer was made of:

10 mM Tris Cl [Sigma], pH 8.0

25 mM EDTA [Sigma], pH 8.0

0.1 M NaCl

Following addition of 250 µl of 10% sodium dodecyl sulfate (SDS) [Sigma], and 100 µl proteinase K [Northumbria Biologicals Ltd] at concentration 10 mg/ml, the samples were mixed with cut off blue tips. Incubation was carried out at 50°C overnight with gentle shaking at 50°C in a hybridisation oven [Techne].

b) DNA extraction

The DNA was extracted and purified twice with an equal volume of buffer-saturated phenol [Aldrich], once with phenol/chloroform, and then once with 24:1 (v/v) chloroform [BDH] : isoamylalcohol [Sigma] mixture. The DNA solution was precipitated with two volumes of ice-cold ethanol and half the original volume of 7.5 M ammonium acetate

[Fisons Scientific Equipment]. DNA was spooled out with a bent pasteur pipette into an Eppendorf™ tube, washed twice in 70% ethanol and dried in vacuum for approximately 15 minutes. The dry DNA pellete was then resuspended in 50 - 200 µl (depending on the size of the pellet) of 10 mM Tris [Sigma] and 1 mM EDTA [Sigma] (TE), pH 7.6, and stored at 4°C

c) Assessment of quality of isolated genomic DNA

To check if DNA was degradated prior to cutting, 2 µl of the DNA sample was mixed with 2 µl of gel loading buffer and loaded on a 1% (w/v) agarose [International Biotechnologies, INC] minigel containing 1 µg/ml ethidium bromide [Sigma]. Gel loading buffer was prepared as follows:

100 mM EDTA, disodium salt [Sigma], pH 8.0
50% (v/v) glycerol [Sigma]
0.1% (w/v) bromophenol blue [BDH]
water to volume.

The gel was run in 1X Tris-borate EDTA (TBE) electrophoresis buffer at 80-100 V for 60 minutes on a minigel apparatus [Bio-Rad]. TBE buffer was made up as 10X concentrated stock solution and stored at room temperature in glass bottles:

109 g Tris base [BDH]
55 g Boric acid [BDH]
40 ml 0.5 M EDTA [Sigma], pH 8.0
distilled water to 1 liter

The quality of isolated DNA was assessed under ultraviolet light (UV).

d) Digestion of DNA with restriction endonuclease

The DNA samples were digested with the restriction enzyme Hae III according to the manufacturer's specifications. Volumes of the components used for digestion of DNA are shown in Table 2.5.5.1.

Table 2.5.5.1. Volumes of the components used for digestion of DNA by restriction enzyme. [a]10X REACT 2 buffer consisted of 0.1 ml of 5 M NaCl, 0.06 ml of 1 M Tris HCl (pH 7.4), 0.06 ml of 1 M $MgCl_2$, 0.01 ml of 1 M dithiothereitol (DDT), 0.67 ml of distilled water.

	60 µl of DNA sample
	8 µl of 10x salt REACT 2[a] [Gibco BRL]
	8 µl of BSA [Sigma], 1 mg/ml
	4 µl of Hae III enzyme, 10 U/µl [Gibco BRL]
Total	80 µl

After overnight digestion at 37°C, completeness of digestion was checked on a minigel with ethidium bromide fluorescence (see above).

e) Purification of digested DNA

In order to eliminate the salt and enzyme from the digested DNA the DNA samples were purified using phenol/chloroform (X1) and chloroform (X1) extraction. DNA was precipitated with 3 volumes of ice cold 100% ethanol with 1/10 volume of 2.75 M sodium acetate. In order to pellet DNA, the samples were spun at 13,000 rpm for 5 minutes in a minifuge Model Microcentaur [MSE Scientific Instruments]. The pellets

were washed twice in 70% ethanol, and spun briefly at 13,000 rpm in a minifuge. DNA pellete was dryed in vacum for 15 minutes, recovered in 20 µl of TE, and stored at 4°C.

f) Measuring of concentration of DNA

The DNA concentration of the samples was measured on a DNA fluorimeter Model TDK-100 [Hoefer Scientific Instruments]. To calibrate the apparatus, standard calf thymus DNA [Clontech Laboratories Inc.] was used at concentrations of 100, 250, and 500 µg/ml. 2 µl of sample was added to 2 ml of working dye solution which consisted of 10 mM Tris Cl, pH 8.0; 0.1 M NaCl; 1 mM EDTA, pH 8.0 (TNE), and Hoechst dye (0.1 µg/ml).

2.5.5.2. Gel electrophoresis and Southern blotting

Separation of DNA fragments was performed by electrophoresis on a 0.8% (w/v) agarose gel, followed by denaturation and DNA capillary transfer to a nitrocellulose membrane.

a) Preparation of agarose gel

2.4 g of powdered agarose [International Biotechnologies, INC] was added into an Erlenmeyer flask with 300 ml of 1X TBE and heated in a microwave oven until the agarose had dissolved. The solution was cooled to approximatelly 60°C, and warm Milli Q (MQ) water added to original level to compensate for evaporation during boiling.

A horizontal gel tray was leveled, the agarose poured and a gel comb inserted. Once the gel has hardened, the gel was overlayed with a sufficient 1X TBE buffer and the comb was carefully withdrawn.

b) Loading DNA samples and running gel

Before loading the gel, the DNA concentration in all samples was standardized to 500 µg/ml by mixing the samples with gel-loading buffer (see section 2.5.4.1.c). Lambda DNA ladder marker (cut with restriction endonuclease EcoR I) [Gibco] and undigested plasmid DNA (homologous to the RNA probe) were run in parallel with the DNA samples. Lambda phage DNA digested with EcoR I gave a useful set of bands: 40; 90; 112; 116; 132; and 180 Kbp. In order prepare the lambda DNA ladder for 2 wells (1 for either side of the gel) the following components were mixed:

10 µl lambda DNA (concentration 500 µg/ml)
1 µl of EcoR I enzyme
2 µl of 10X salts
7 µl of distilled water

Mixture was incubated at 37°C for 1.5 - 2 hours. Following incubation, the digest was mixed with 20 µl of glycerol dye and split between two wells.

The wells were loaded with equal amounts of DNA sample and dye ensuring that the volume of the dye was 1/3 of the load. After the samples, lambda and uncut plasmid DNA were loaded, the gel was left for 10 minutes before running in order to allow DNA to equilibrate with buffer. Electrophoresis conditions for separation of DNA fragments were given in Table 2.5.5.2.

Table 2.5.5.2. Electrophoresis condition for separation of DNA fragments by agarose gel for DNA fingerprint analysis. [a]The gel was run first at 24 V for 48 hours (when the buffer was changed), and after run at 36 V for another 24 hours.

Format	horizontal
Run time	72 hrs
Gel concentration	0.8%
Gel size	20 x 20 cm
Gel thickness	4-6 mm
Gel buffer	1X TBE
Running buffer	1X TBE
Sample volume	20 µl
Voltage	24 V (36 V)[a]
Temperature	ambient

The same batch of electrophoresis buffer was used in both the electrophoresis tank and for preparing the gel (100 mM Tris borate, 1 mM EDTA, pH 8.3). This is because small differences in ionic strength and pH creates fronts in the gel which can affect the mobility of DNA fragments. After 48 hours the whole running buffer was replaced with 1X TBE containing 1 µg/ml of ethidium bromide. This was necessary for visualizing the migration of lambda DNA ladder. The migration distance of each ladder was measured under the UV and recorded.

c) Capillary transfer of DNA by Southern blotting

Following electrophoresis, DNA was depurinated by soaking the gel in 0.25 M HCl for 20 minutes on a shaking tray, and denatured by placing in a bath of 0.5 N NaOH and 1 M NaCl (Gel Soak 1) for 45 minutes at

room temperature on a moving platform. The gel was then neutralized by bathing in 0.5 M Tris-HCl pH 7.4, and 3 M NaCl (Gel Soak 2) for 45 minutes at room temperature on a moving platform.

The DNA was transferred from the gel by DNA capillary transfer as described by Southern (1975) (Figure 2.5.5.1.). Transfer was performed in a deep dish with 1 liter of 10x SSC buffer (01.5 M NaCl, 0.15 M sodium citrate). Stock SSC solution (20X) was prepared by dissolving 175.3 g NaCl (3M) and 88.2 g Na_3 citrate·$2H_20$ in 800 ml of distilled water. The pH of

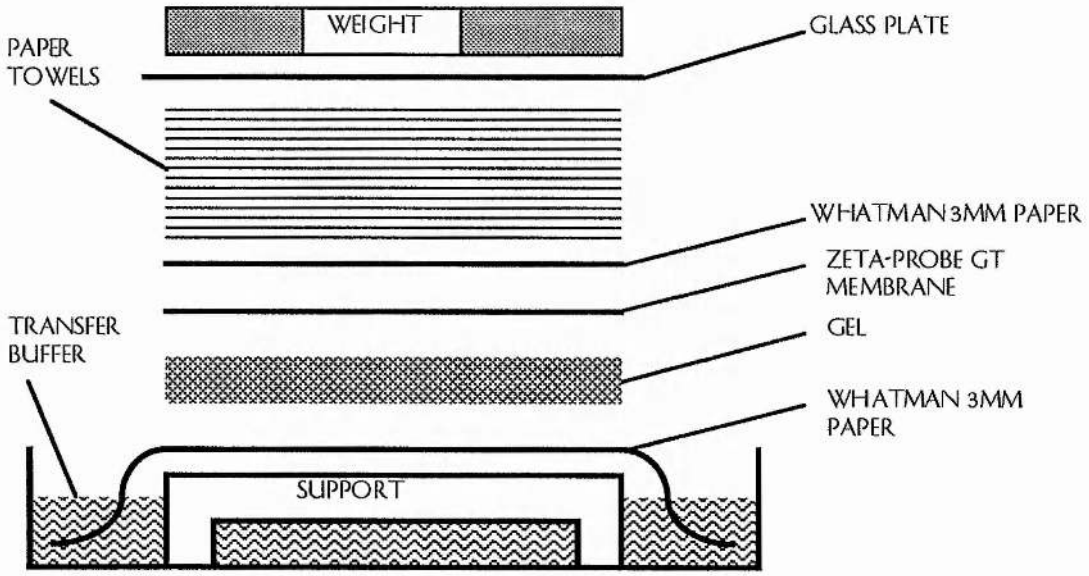


Figure 2.5.5.1. Capillary transfer of DNA from agarose gel onto Zeta-Probe GT membrane.

the solution was adjusted to 7.0 with a few drops of10 N Na OH. The volume was adjusted to 1 liter with distilled water and solution was sterilized by autoclaving.

Five sheets of Whatman 3MM blotting paper were cut (four sheets approximately the same size as the gel and one sheet twice as long as the

gel). One sheet of Zeta-Probe GT nitocellulose membrane [Bio-Rad] was cut to the exact size of the gel and soaked for 5 minutes in distilled water before using. A wick of Whatman 3MM paper was laid first over the glass so both ends reach into the transfer buffer. About 1 L of 10X SSC was poured over the wick and rolled out with a 10 ml pipette to remove any trapped air bubbles.

Two more sheets of the pre-cut 3MM Whatman paper were then placed on the wick, and the gel placed on it. Saran Wrap was spread along all four sides of the gel. This ensured capillary action only through the gel. After flooding the gel surface with buffer, the pre-wetted Zeta-Probe GT membrane was placed on it, and trapped air bubbles removed as above. The two remaining sheets of 3MM Whatman paper, cut slightly smaller then the membrane, were soaked in 10X SSC and placed on the surface of the blotting membrane.

Paper towels (about 15 cm high) were stacked over the Whatman paper being careful to avoid any chance of a short circuit. In order to keep pressure, a glass plate and weight cones were placed on top of the towel stack.

In order to keep up steady capillary action, the damp towels were changed every 5 minutes for the first 15 minutes, thereafter every 15 minutes for the first hour. The transfer was continued overnight for further 20 hours.

After the transfer was completed, the membrane was separated from the gel using blunt forceps, rinsed briefly in 2 x SSC to remove debris. The membrane was then air dried on a piece of filter paper, and baked in a preheated vacuum oven at 80°C for 30 minutes, and stored dry between two filter papers in a plastic bag at room temperature until use.

2.5.5.3. Cloning and radiolabelling of RNA probe

a) Cloning of minisatellite sequences into vector

An RNA probe was synthesized from a specific DNA template. This template was inserted downstream from a specific bacteriophage transcription promoter (subcloning of minisatellite sequence into vector was performed by Dr. D. Parkin, Nottingham). Transcribable vectors pSPT 18 and pSPT 19 [BCL] (Figure 2.5.5.2.) which contained RNA polymerase promoters from T7 and SP6 phages flanking the pUC 18 multiple cloning site were selected. They also conferred ampicillin resistance. Minisatellite region of 33.6 and 33.15 were liberated by digestion of M13 RF DNA with Hind III and EcoRI, and purified by electroelution. Minisatellite regions were then cloned into the Hind III/EcoRI site of vectors pSPT 18.6 and 19.6 to yield 4 recombinants (pSPT 18.6, 19.6, 18.15, and 19.15) permitting either strand of either probe to be prepared, by selecting T7 or SP6 polymerase for transcription. The recombinants were transferred by the $CaCl_2$ method into *E. coli.* DH1 strain, and recombinants were selected by ampicillin resistance.

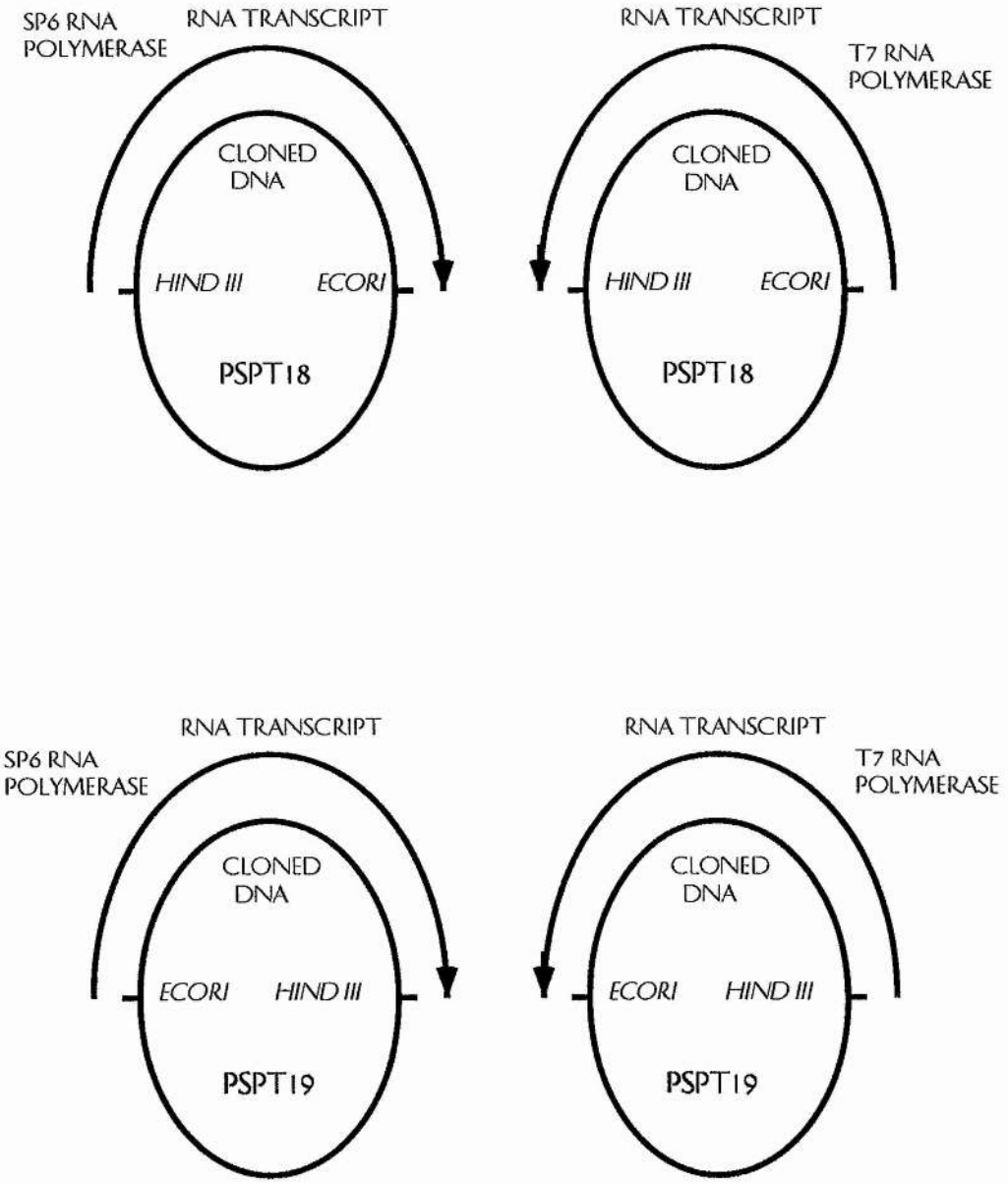


Figure 2.5.5.2. Principle of SP6/T7 transcription. Transcription of the (+) and (-) strand of the cloned DNA is possible with SP6 RNA polymerase and T7 RNA polymerase in the same vector or with only one of the RNA polymerases in different vectors.

b) Isolating plasmid DNA from E. coli

Some *E. coli* bacteria were taken from glycerol stock (freezer) with a sterile inoculation wire and incubated overnight on Luria Broth (LB) ampicillin (50 µg/ml) plates at 37°C to produce single colonies. A single bacterial colony was streaked onto a fresh LB ampicillin plate and incubated again overnight at 37°C. A single colony was then removed from this second plate and inoculated into 3 ml of LB ampicillin broth (a rich growth medium) and incubated overnight at 37°C with gentle agitation.

One liter of LB broth was prepared as follows: 10 g Bactotryptone [Difco], 5 g Bacto yeast extract [Difco], and 10 g NaCl were mixed in water and pH was adjusted to 7.0 using 5 N NaOH. Following adding 15 g Bacto-agar, the solution was autoclaved. Molten media were allowed to cool to 55°C before adding ampicillin to a final concentration of 35-50 µg/ml. Ampicillin was prepared as 25 mg/ml stock solution in water and stored in aliquots at -20°C.

To isolate the plasmid DNA from the bacteria the method described by Jones & Schofield (1990) was employed. 1.5 ml of bacterial suspension was pelleted in an Eppendorf™ tube by centrifuging for 1 minute at 13,000 rpm in a minifuge. The supernatant was removed completely and the bacterial pellet resuspended in 180 µl solution of 50 mM glucose, 25 mM Tris-HCl, pH 8.0, and 10 mM EDTA (GTE). A freshly prepared solution of 0.2 M NaOH and 1% SDS was used as a lysis mix. Following addition of 360 µl of lysis mix to the cells, the tubes were inverted 10 times and then incubated on ice for 5 minutes.

To precipitate the chromosomal DNA and proteins, 270 µl of 3 M potassium acetate (pH 4.8) was added, inverted 10 times and then the mixture was placed on ice for further 5 minutes.

After spinning at 13,000 rpm for 5 minutes in a minifuge at 4°C, the supernatant which contained the plasmid DNA was transferred to a clean tube. One volume (800 µl) of absolute ethanol was added, vortexed briefly, and immediately spun at 13,000 rpm for 5 minutes. The resulting supernatant was discarded and the pellet (the plasmid) was washed with 1 ml of 70% ethanol, before a final centrifugation at 13,000 rpm for 2 minutes. The plasmid pellet was dried in vacuum for 10 minutes. The pellet was resuspended in 20 µl of sterile TE.

c) Radiolabelling of RNA probe

Linearizing the plasmid DNA. The plasmid DNA was linearized by digestion with a suitable restriction enzyme. Into a sterile tube the following components were added:

3 µl (1 µg) of uncut plasmid DNA
1 µl enzyme (EcoR I [Gibco BRL] for pSPT 19.6, and
Hind III [Gibco BRL] for pSPT 18.15)
0.6 µl 10X salt solution (REACT 3) [Gibco]
1.4 µl sterile distilled water

The mixture was incubated for 1.5 hours in a 37°C water bath.

Transcription reaction. To radiolabel RNA probe, the protocol described by Carter *et al.* (1989) was followed. Transcription reaction was perfomed by addition of components as shown in Table 2.5.5.3.

Table 2.5.5.3. Volumes of components used in transcription reaction for radiolabelling RNA probe. [a]From Promega Riboprobe Gemini II kit. [b]Transcription buffer (5X) consisted of 200 mM Tris-HCl pH 7.5, 30 mM $MgCl_2$, 10 mM spermidine, 50 mM NaCl.

6 µl linearized plasmid DNA

4 µl 5X transcription buffer[ab]

3 µl NTPs (10 µM each ATP, GTP, UTP, 1 µM CTP)[a]

2 µl DTT (100 mM)[a]

1 µl RNAsin (25 U/µl) [Amersham]

3 µl ^{32}P CTP (400 Ci/mmol) [Amersham]

1 µl T7 RNA polymerase.(20 U/µl) [Pharmacia]

Total 20 µl

This mixture was vortexed, spun briefly and incubated for 1 hour at 37°C. The reaction was stopped by the addition of an equal volume (20 µl) of a mixture of 0.9% (w/v) blue dextran [Sigma], 0.03% (w/v) bromocresol purple [BDH], and 20 mM EDTA (Nick stop mix).

Removal of unincorporated nucleotides. The unincorporated nucleotides were removed on a 1 ml column Sephadex G-50 [Pharmacia] equilibrated with TE. The bottom of a 1 ml disposable syringe was plugged with glass wool. The syringe was filled with the column resin and placed into a polypropylene tube that is suitable for centrifugation. In order to pack the column, spinning was performed at 3,000 rpm for 3 minutes in a desktop centrifuge. The sample tube was rinsed with 2 volumes of TE, reloaded into the column, and spun at 3,000 for 3 minutes. All liquid (the labelled RNA probe) at the bottom of the tube was collected. Relative radioactive incorporation was measured in a liquid scintilation counter Model Tricarb 1600 TR [Canberra Packard].

2.5.5.4. Hybridization and visualization

The prehybridisation was carried out overnight in hybridization tubes at 65°C in a solution of 1X SSC, 2% SDS, and 1% bovine lacto transfer technique optimizer solution (BLOTTO). The BLOTTO stock solution (10%) was prepared by dissolving 10 g non-fat dried milk [Marvel] in 100 ml of water, followed by addition of 0.2 g sodium azide and 10 µl diethyl pyrocarbonate (DEPC).

Prehybridization was followed by hybridisation in the same solution used for prehybridisation however with the addition of 6×10^7 cpm ^{32}P-labelled RNA probe (pSPT 19.6). Hybridization was carried out in the hybridization chambers at 65°C for 16 hours.

Following the hybridization the membrane was washed repeatedly in 1X SSC and 0.1% SDS at 65°C until a blank control filter showed only background radiation levels.

After washing, the damp membrane was wrapped in Saran Wrap and exposed to two Kodak X-ray films (either side of the membrane) for 2 days with two intensifying screens followed by 19 days without screens at -70°C. One film was developed after 2 days and the other after 21 days.

2.6. Characterization of the tumour cell lines

2.6.1. Plating efficiency and survival curves

2.6.1.1. Plating efficiency (PE)

Cells were trypsinized (see section 2.1.1.) and single cell suspensions were prepared by pipetting. The cell concentration was determined using a Coulter counter model ZM [Coulter Electronics Ltd] with the following settings: threshold 20.0; attenuation 32; aperture current 0.7 mA. The cells were plated out as a single cell suspension in 10 ml of medium containing 7% FCS, at low cell densities into 90 mm diameter cell culture grade plastic dishes [Nunc]. The cultures were incubated for 14 days at 37°C in a humidified gas atmosphere with 5% CO_2. Following incubation, the cultures were washed with PBS, fixed with methanol [Fisons Scientific Equipment] for 10 minutes, air dried, and stained with 10% (v/v) Giemsa [BDH] for 20 minutes. Colonies with more than 50 cells were counted.

The plating efficiency (PE) which can de defined as the percentage of the untreated cells that grow into macroscopic colonies was calculated by the following equation:

$$PE = \frac{\text{Number of colonies counted}}{\text{Number of cells seeded}} \times 100 \quad (\%)$$

2.6.1.2. Survival assay

Figure 2.6.1.1. shows the schematic protocol used to generate survival curves. Cells were trypsinized and the cell concentration was determined using a Coulter counter as described in section 2.6.1.1. Cells in suspension were exposed to graded doses of γ-irradiation. The number of irradiated cells was chosen so as to produce approximately 60 colonies per dish. The treated cells were then plated into 90 mm diameter cell culture grade plastic petri dishes [Nunc]. The plates were incubated for 14 days in a humidified gas atmosphere containing 5% CO_2 at 37°C.

Following incubation, the cultures were washed with PBS, fixed with methanol for 10 minutes, air dryed, and stained with 10% (v/v) Giemsa [BDH] for 20 minutes. Colonies with more than 50 cells were counted.

The surviving fraction (SF) which can be described as the proportion of treated cells that can give rise macroscopic colonies was calculated as follows:

$$SF = \frac{\text{Number of colonies}}{\text{Number of cells seeded} \times PE/100}$$

a) Statistical analysis

Comparison of radiosensitivity between the tumour cell lines and parent HTori3 cells was performed using a two way analysis of covariance (Ancova)

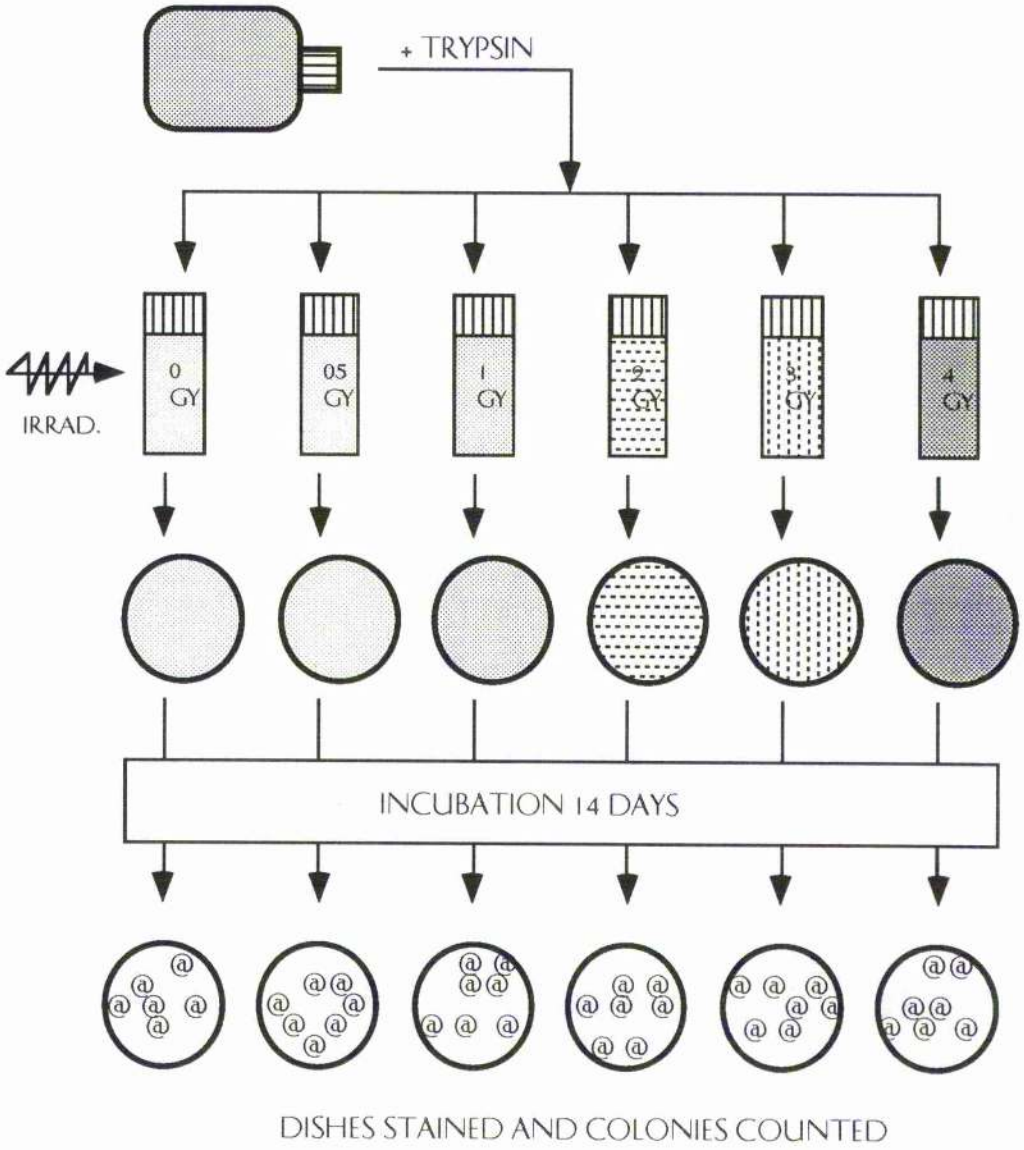


Figure 2.6.1.1. The cell culture technique used to generate cell survival curves.

2.6.2. Detection of *ras* mutations in the tumour cell lines

Screening for *ras* oncogene mutations in the tumour cell lines was performed using a rapid nonradioactive method (Jiang *et al.*, 1989). Polymerase chain reaction (PCR) amplification of human H-*ras* and K-*ras* first exon sequences is followed by specific restriction enzyme analysis to detect either endogenous or primer-mediated restriction fragment length polymorphisms (RFLP).

2.6.2.1. The basic principles of PCR

Although the principle of the polymerase chain reaction (PCR) was described in details in early 1970s (Kleppe *et al.*,1971; Panet & Khorana, 1974), the first PCR reaction was performed and named in 1985 by Mullis and colleagues (Mullis & Faloona, 1987). The PCR technique has enabled us to produce enormous numbers of copies of a *specified* DNA sequence without resorting to time-consuming cloning techniques.

The principle behind PCR is quite simple (see Figure 2.6.2.1.). The technique consists of three stages: denaturation, annealing and extension, which are repeated over 20 or more cycles. This technique exploits certain features of DNA replication. DNA polymerase uses single-stranded DNA as a template for the synthesis of a complementary new strand in the 5' to 3' direction. Single-stranded DNA can be produced by heating double-stranded DNA to temperatures near boiling. DNA polymerase also requires a small section of double-stranded DNA to initiate ("prime") synthesis. Short oligonucleotide sequences (primers) are designed to flank the region of DNA that is to be amplified. Since a primer is

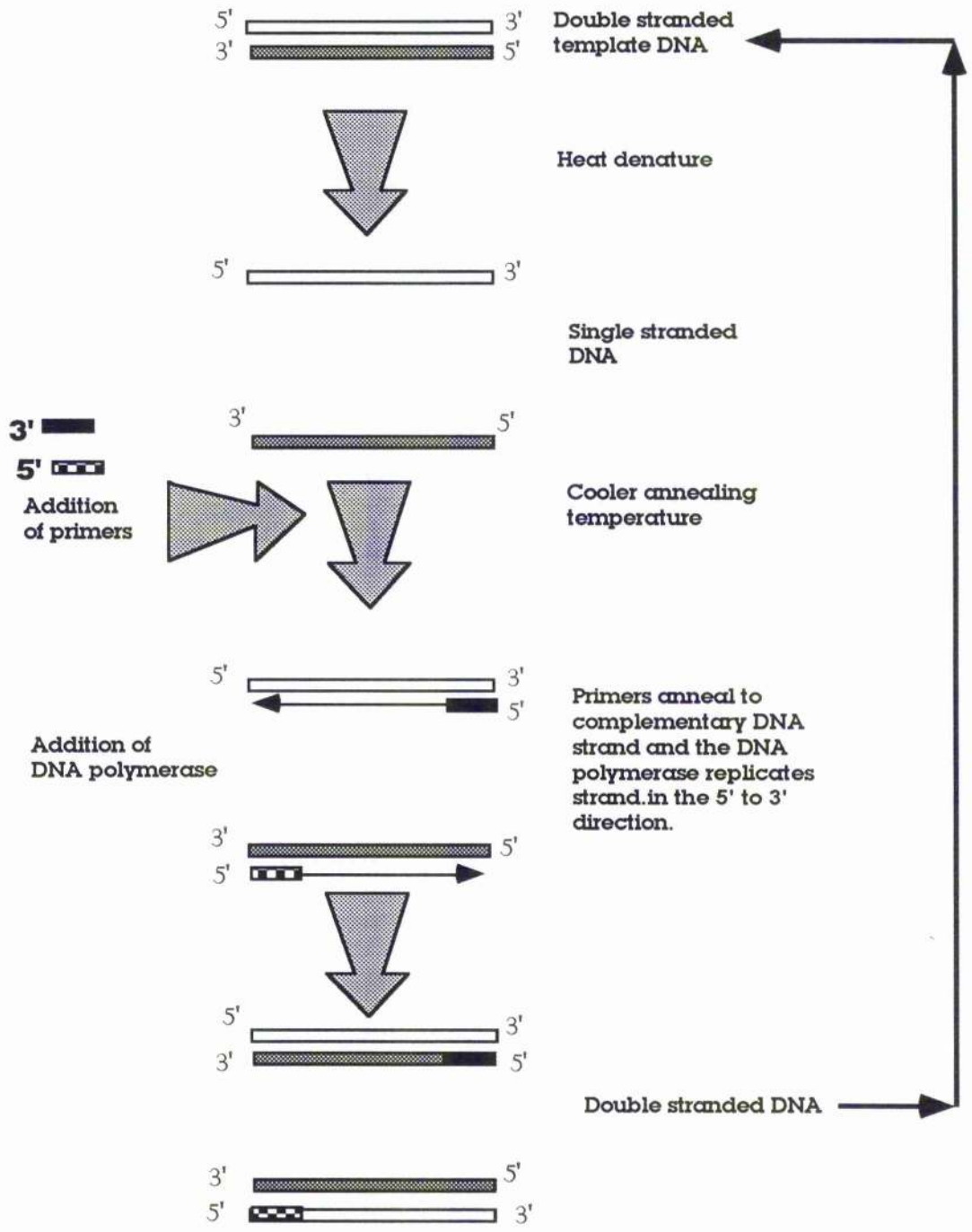


Figure 2.6.2.1. Schematic diagram of PCR reaction. When primer 1 and 2 are added, they anneal to complementary strands of DNA and two new strands are synthesized by *Taq* polymerase. If the process is repeaed, both the sample and newly sinthesized strands can serve as templates, leading to an exponential increase of product (redrawn from Read, 1990).

provided for each DNA strand, both DNA strands serve as templates for synthesis

The double-stranded template DNA is denatured by heating at 94 - 95°C resulting in formation of single DNA strands. Cooling to 40 - 60°C allows the primers to anneal. The mixture is subsequently heated to the optimal temperature for the polymerase (usually 72°C). This allows the polymerase to copy the complementary strands. The newly synthesized strands of DNA extend beyond the position of the primer on the opposite strand. Therefore new primer binding sites are generated on each newly synthesized DNA strands.

The mixture is heated again to separate the original and newly synthesized strands, which are then available for primer annealing, DNA synthesis, and strand separation. Most PCR amplifications consists of between 20 and 30 such cycle. A final incubation step at the extension temperature results in fully double-stranded DNA fragments.

Initially synthesis goes beyond the sequence complementary to the other primer, but with each cycle of heating and cooling, the number of the fragments in the region flanked by each primer increases almoust exponentially, while longer fragments accumulates only in a linear fashion, provided that the amount of starting DNA is present in limiting quantities.

The use of PCR had had very limited applications until heat-stable DNA polymerase become available. Since "Klenow" polymerase, originally used in PCR reactions (Mullis & Faloona, 1987), is a heat sensitive enzyme, it had to be replenished after each denaturation step. The isolation of the thermally stable *Taq* polymerase (from eubacteria

Thermus aquaticus) allowed just one aliquot to be added at the start of the procedure (Saiki *et al.*, 1985; Powell *et al.*, 1987; Chehab *et al.*, 1987).

2.6.2.2. Oligonucleotide primers and PCR amplification

Oligonucleotide primers for PCR amplification were synthesized by Oswel DNA Service, University of Edinburgh. The base sequences of the primers (Jiang *et al.*, 1989) used for amplification of H-*ras* codon 12 sequences, and K-*ras* codon 12 and codon 13 are shown in Figure 2.6.2.2.

The primers used for amplification of H-*ras* codon 12 sequences spanned two endogenous MspI sites (CCGG). The first, one nucleotide downstream of H5', located in the intron before exon 1, was used as a positive control for MspI cleavage. The second site was polymorphic for H-*ras* mutants at either of the first two positions of codon 12 (Figure 2.6.2.3.).

PCR strategy for amplification of K-*ras* first exon utilized the fact that mismatched primers can effectively be used for PCR amplification (Cohen & Levinson, 1988). Mismatched primers were designed introducing a single nucleotide substitution in both primers (Figure 2.6.2.2.). A single C nucleotide was incorporated at the first position of codon 11 of 5' end primer (K5') creating a Bst NI recognition site (CCTGG). This Bst NI cleavage site was absent in amplified K-*ras* segments mutant at either of the first two positions of codon 12 (Figure 2.6.2.4.). A single G nucleotide was also introduced into the 3' end primer (K3') as a positive control for Bst NI cleavage.

Mutations at codon 13 of K-*ras* were screened by digestion of PCR amplified fragments with the restriction endonuclease Hph I (Figure

2.6.2.4.). K-*ras* codon 13 mutations involve a substitution by an A residue at the second position, resulting in the replacement of glycine by aspartic acid in the oncogenic protein. This substitution created the Hph I restriction site (GGTGA) which can not be "recognized" by the enzyme when a mutation is present.

Primer A: H-*ras* 5': 5' GAG ACC CTG TAG GAG GAC CC 3'

Primer B: H-*ras* 3': 5' GGG TGC TGA GAC GAG GGA CT 3'

Primer C: K-*ras* 5': 5' ACT GAA TAT AAA CTT GTG GTA GTT GGA CCT 3'

Primer D: K-*ras* 3': 5' TCA AAG AAT GGT CCT GGA CC 3'

Figure 2.6.2.2. The base sequences of the oligonucleotide primers used in the PCR amplification of first exon of H-*ras* and K-*ras* oncogenes in the tumour cell lines (sequences taken from Jiang *et al.* 1989). The nucleotide substitutions introduced into K5' and K3' primers to create Bst NI restriction sites are underlined.

High molecular weight DNA was prepared as described in section 2.5.4.1. The only difference was that the DNA had been dissolved in 10 mM Tris Cl (pH 7.6) and 0.1 mM EDTA (pH 8.0). Because the optimal concentration of Mg^{++} ions is quite low (1.5 mM), it is important that the preparation of template DNA does not contain high concentrations of chelating agents such as EDTA.

Reaction mixtures for amplification of H-*ras* and K-*ras* sequences were prepared as shown in Table 2.6.2.1. and Table 2.6.2.2., respectively. PCR buffer, $MgCl_2$ solution, *AmpliTaq* DNA Polymerase, and Deoxynucleoside Triphosphates (dNTPs) were supplied from Perkin Elmer.

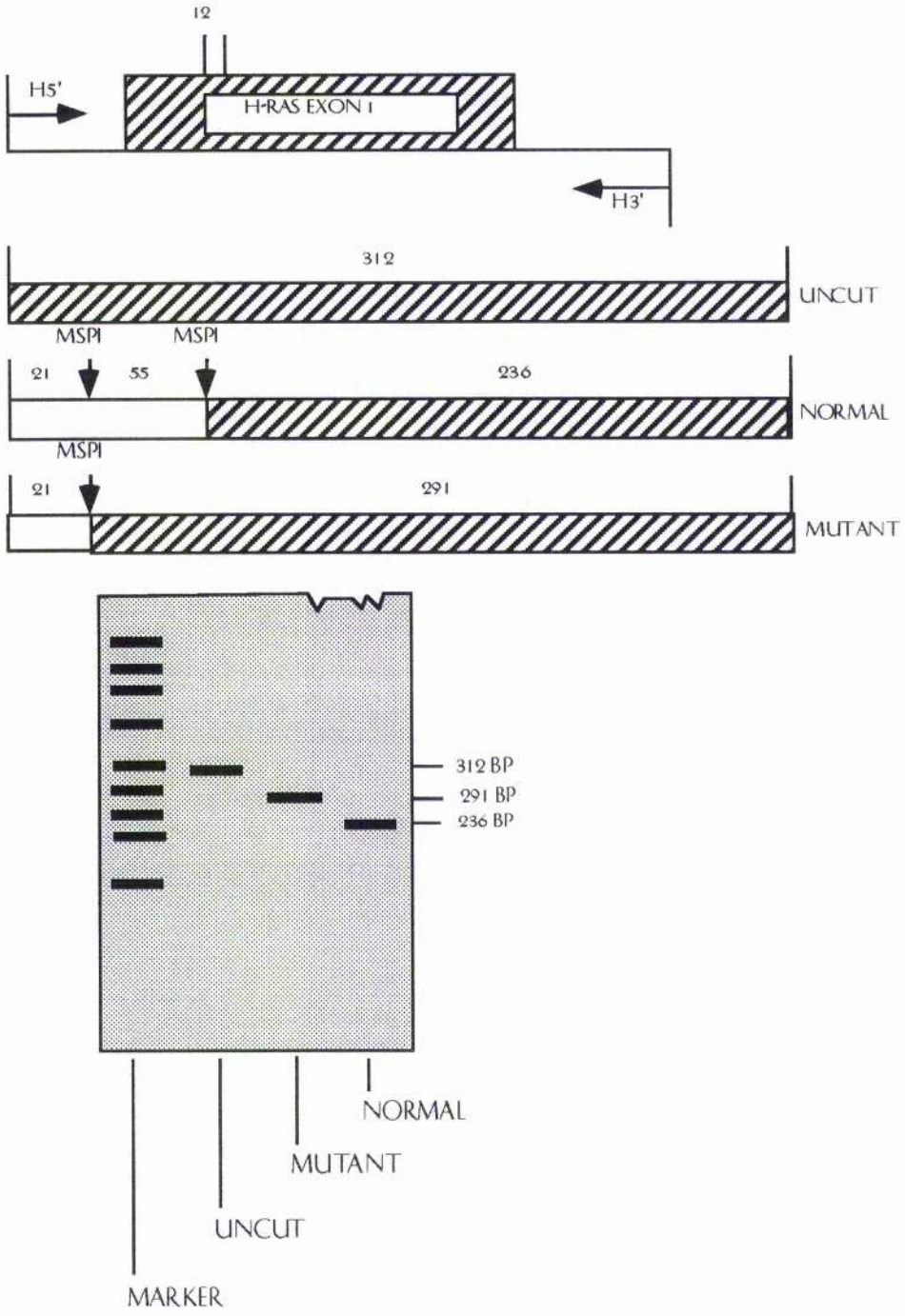


Figure 2.6.2.3. PCR amplification strategy for H-*ras* exon 1 sequences.

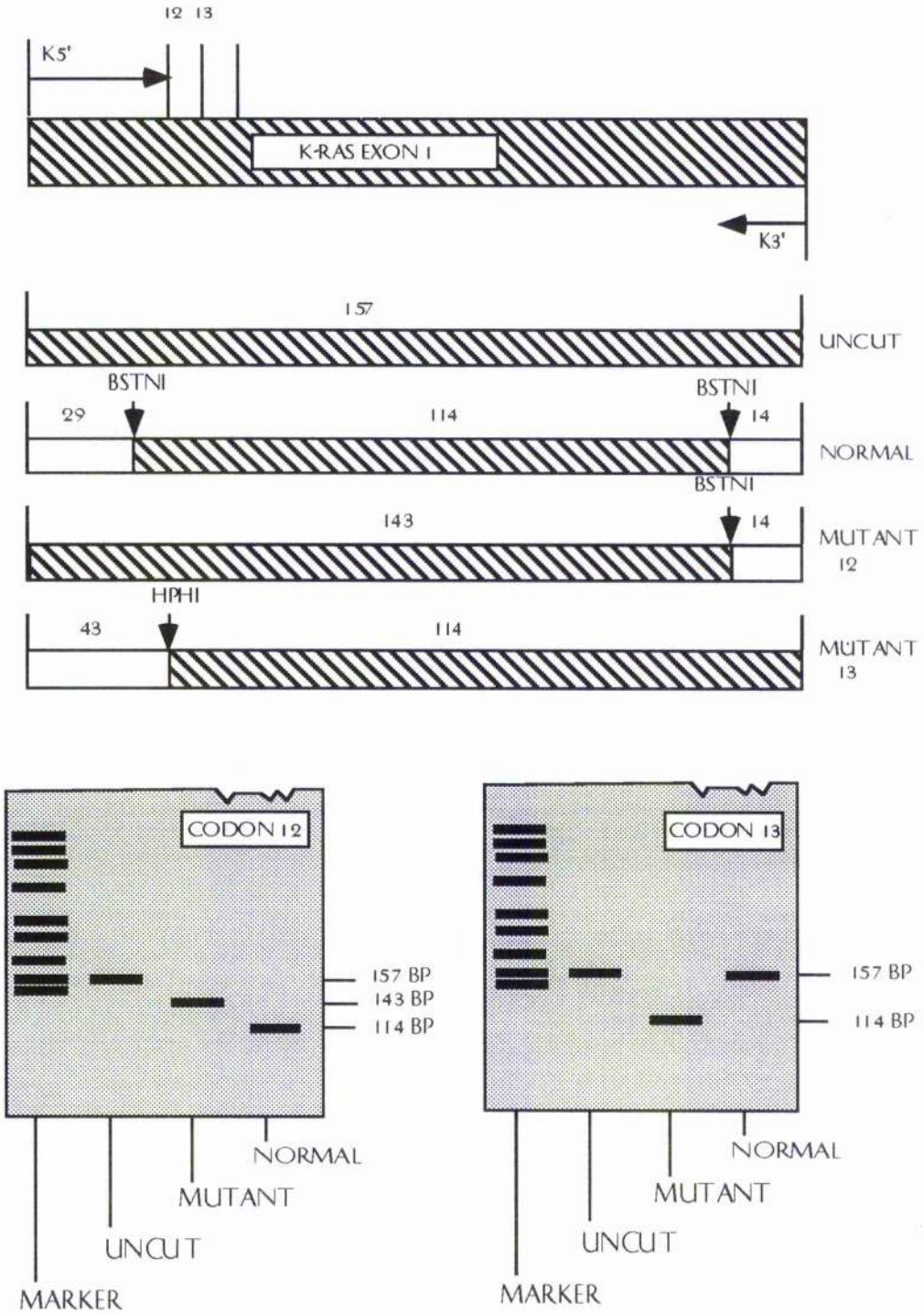


Figure 2.6.2.4. PCR amplification strategy for K-*ras* exon 1 sequences.

Table 1.6.2.1. Reaction mixtures for amplification of H-*ras* exon 1. [a]PCR reaction buffer consisted of100 mM Tris-HCl, pH 8.3; 500 mM KCl; 1.5 mM Mg Cl2; 0.01% w/v gelatin; [b] dNTPs were prepared as a daily working solution with 1.25 mM each dNTP; [c] stock solution of primer A and B were prepared previously as equimolar mixture of 0.04 nm/5 µl each primer.

Component	Conc. of stock	Added volume	Final concentr.	Addition Order
PCR buffer[a]	10X	10 µl	1X	2
dATP stock	10 mM	2 µl	200 µM }	
dCTP stock	10 mM	2 µl	200 µM }	3[b]
dGTP stock	10 mM	2 µl	200 µM }	
dTTP stock	10 mM	2 µl	200 µM }	
$MgCl_2$ solution	25 mM	4 µl	1 mM	5
Primer A	0.04 nm/5µl	5 µl	400 nM }	4[c]
Primer B	0.04 nm/5µl	5 µl	400 nM }	
DNA template	1 µg/10 µl	10 µl	1 µg/100 µl	6
Taq polymerase	5 U/µl	0.5 µl	2.5 U/100 µl	7
Distilled water	-	57.5 µl	-	1
Total		100 µl		

All components were added into sterile 0.5 ml reaction tubes [Treff AG] and quickly spun down in a microcentrifuge. The reaction mixture was then overlaid with 50-100 µl of mineral oil [Sigma]. This was to prevent evaporation of the water at high temperature during amplification. Amplification was carried out in a Techne Gene E thermocycler for 30 cycles under the conditions given in Table 2.6.2.3.

Table 1.6.2.2. Reaction mixtures for amplification of K-*ras* codon exon 1. [a] PCR reaction buffer consisted of 100 mM Tris-HCl, pH 8.3; 500 mM KCl; 1.5 mM Mg Cl2; 0.01% w/v gelatin; [b] prepared daily as a mix of 1.25 mM of each dNTP, added 16 µl per reaction; [c] stock solution of primer C and D were prepared previously as equimolar mixture of 0.04 nm/5 µl each primer

Component	Conc. of stock	Added volume	Final Concentr.	Addition Order
PCR buffer[a]	10X	10 µl	1X	2
dATP stock	10 mM	2 µl	200 µM }	
dCTP stock	10 mM	2 µl	200 µM }	3[b]
dGTP stock	10 mM	2 µl	200 µM }	
dTTP stock	10 mM	2 µl	200 µM }	
$MgCl_2$ solution	25 mM	8 µl	2 mM	5
Primer C	0.04 nm/5 µl	5 µl	400 nM }	4[c]
Primer D	0.04 nm/5 µl	5 µl	400 nM }	
DNA template	1 µg/10 µl	10 µl	1 µg/100 µl	6
Taq polymerase	5 U/µl	0.5 µl	2.5 U/100 µl	7
Distilled water	-	53.5 µl	-	1
Total		100 µl		

Each cycle consisted of the following steps: template denaturation (melting), primer reannealing, extention (elongation). Reaction mixtures were subjected to an initial template melting step for before the start of the cycling.

Table 2.6.2.3. The conditions for PCR amlification of H-*ras* and K-*ras* sequences. [a]NC = number of cycles

		Each of 30 cycles			
	Initial step	Melt	Anneal	Extend	Final step
Time	1 min	1 min	2 min	3 min	5 min
Temp.	94°C	94°C	55°C	72°C	72°C
NC[a]	1	30	30	30	1

After completing 30 cycles, the final extension step was applied in order to allow to complete extention of all strands. After amplification, the samples were stored at 4°C and withdrawn as required through the mineral oil.

2.6.2.3. The restriction enzyme analysis

a) Restriction enzyme analysis of H-ras codon 12

Digestion of amplified H-*ras* exon 1 sequences (20 µl aliquots) were performed with 40 units of restriction endonucleases Msp I [Sigma] for 2-3 hours at 37°C. Digestion mixture were prepared as shown in Table 2.6.2.4.

Table 2.6.2.4. Digestion of PCR amplified H-*ras* exon 1 sequences. [a] Upon dilution to 1X Pelette Buffer Blue [Sigma] consisted 10mM Tris-HCl, pH 7.9, 10 mM $MgCl_2$, 50mM NaCl, 1 mM DTT.

PCR amplified samples	20 µl
10X Palette Buffer Blue[a]	4 µl
Msp I enzyme	4 µl
Distilled water	12 µl
Total	40 µl

Amplified normal H-*ras* fragments were 312 nucleotides long (visible by agarose gel electrophoresis and ethidium bromide staining). Digestion with the restriction endonuclease MspI resulted in a band of 236 nucleotides and 2 smaller bands of 55 and 21 nucleotides (usually not seen in these gels) (Figure 2.6.2.3.). A larger band of 291 nucleotides indicated the detection of mutation at codon 12. The T24 human bladder carcinoma cell line, homozygous for the H-*ras* codon 12 mutant valine allele was used as a positive control for H-*ras* codon 12 mutations.

b) Restriction enzyme analysis of K-ras codon 12 and codon 13

Amplified K-*ras* exon 1 samples were digested with restriction endonucleases either BstN I or Hph I. BstN I was used in order to analyse for K-*ras* codon 12 mutants, and Hph I for analysis of K-*ras* codon 13 mutants. PCR sample aliquots were digested for 2-3 hours with 40 units of the restriction enzyme BstN I [Sigma] at 60°C or 20 units of Hph I [Sigma] at 37°C. Mixture for digestion were prepared as shown in Table 2.6.2.5. and Table 2.6.2.6.

Table 2.6.2.5. Digestion of PCR amplified K-*ras* exon 1 sequences for analysis of K-*ras* codon 12. [a] Bovine Serum Albumin solution [Sigma], required for full activity of BstN I, was used in concentration of 1 mg/ml

PCR amplified samples	20 µl
10X Palette Buffer Blue	4 µl
BSA[a]	4 µl
BstN I enzyme	4 µl
Distilled water	8 µl
Total	40 µl

PCR amplification of K-*ras* exon 1 sequences generated a DNA fragment of 157 nucleotides (visible after ethidium bromide staining). Upon digestion with BstN I enzyme, fragments encoding normal codon 12 sequences were cleaved twice, resulting in a band of 114 nucleotide and smaller bands of 29 and 14 nucleotides (Figure 2.6.2.4.). Fragments containing mutations at this sequence were not "recognized" by the enzyme and therefore were cleaved once. This resulted in a band of 143 nucleotides and a smaller band of 14 nucleotides.

In order to screen for K-*ras* codon 13 mutations, PCR fragments were digested with Hph I. Normal fragments remained undigested (band of 157 nucleotides), while codon 13 aspartic acid mutations were cleaved once resulting in a band of 114 nucleotides and smaller band of 43 nucleotides (Figure 2.6.2.4.).

Table 2.6.2.6. Digestion of PCR amplified K-*ras* exon 1 sequences for analysis of K-*ras* codon 13. [a] Upon dilution to 1X Palette Buffer Green [Sigma] consisted 20 mM Tris-Acetate, pH 7.9, 10 mM $Mg(OAc)_2$, 50 mM KOAc, 1 mM DTT.

PCR amplified samples	20 µl
10X Palette Buffer Green[a]	4 µl
Hph I enzyme	4 µl
Distilled water	12 µl
Total	40 µl

2.6.2.4. Electrophoresis of digested PCR fragments

Digested DNA samples were electrophoresed through either MetaPhor™ agarose (H-*ras* codon 12) or nondenaturing polyacrylamide gel - PAGE (K-*ras* codon 12 and codon 13).

MetaPhor™ agarose [FMC] has the finest resolution capabilities compared to other agarose product and is used for analysis of larger H-*ras* fragments (approximately 300 nucleotides). However, MetaPhor™ agarose approximates the resolution of only low concentration polyacrylamide gels (3.5% to 8%) and this was the reason why PAGE was used for analysis of smaller K-*ras* fragments.

a) MetaPhor™ agarose electrophoresis

Details of a typical electrophoresis conditions for MetaPhor™ gels are given in Table 2.6.2.7. MetaPhor™ agarose [FMC] was weighed and added to the chilled buffer (1X TBE). Dissolving agarose was performend in microwave by several short 20 to 60 heating intervals with gentle swirling between pulses. Solution wa cooled to 60 to 70°C before casting

the gel in a horizontal gel tray. Once the gel is cast, the molten agarose was allowed to cool at room temperature for 30 minutes. The gel was then placed at 4°C for 30 minutes in order to obtain optimal resolution and gel handling characteristics.

Once chilled, the gel was overlayed with a thin layer of buffer (1X TBE), and the comb was removed. The digested DNA samples mixed with appropriate volume of 6X gel loading buffer consisting of the following:

0.25% bromophenol blue [Sigma]
40% (w/v) sucrose [BDH]
water to volume

Electrophoresis were carried out at 80 V at room temperature for 3-4 hours. As a DNA marker, 0.5 µg of ΦX174 Hae III digest was always run in parallel. ΦX174 Hae III digest was prepared for electrophoresis as follows:

0.2 mg of ΦX174 Hae III digest [Sigma] (supplied at concentration 1340 µg/ml in 10 mM Tris-HCl, pH8.0, and 1.0 mM EDTA)
0.05% bromophenol blue [Sigma]
40% w/v sucrose [BDH]
0.1 M EDTA, pH 8.0
water to volume (200 µl).

The mixture was aliquoted (10 µl) into 0.5 ml Eppendorfs and stored at -20°C. 5 µl of the solution was loaded for each agarose gel.

Table 2.6.2.7. Electrophoresis condition for MetaPhor™ agarose gels.

Format	horizontal
Run time	3-4 hrs
Gel concentration	4%
Gel length	20 cm
Gel thickness	4-6 mm
Gel buffer	1X TBE
Running buffer	1X TBE
Sample volume	20 µl
Voltage	4-5 V/cm
Temperature	ambient

After electrophoresis, the gel was stained by submersion in staining solution of 0.5 µg/ml ethidium bromide [Sigma] in 1X TBE. After staining for 30-45 minute at room temperature, gels are viewed and photographed on a ultraviolet light transilluminator.

b) Nondenaturing PAGE electrophoresis

Volumes of reagents used to cast a 1.5 mm thick PAGE gel are shown in Table 2.6.2.8. Appropriate volumes of 30% acrylamide stock solution, 10X TBE, water and 10% ammonium persulfate solution were pipetted into a 100 ml beaker. In order to reduce the chance of air bubbles formation, the solution was deaerated in vacuum for 20 minutes.

The glass plates and spacers were thoroughly washed in detergent and 70% ethanol and rinse in deionized water. The glass plates must be free of grease spots to prevent air bubbles from forming in the gel. The glass plates and spacers were arranged to create watertight seal so that the

unpolymerized gel solution does not leak out. In addition to prevent leakage of acrylamide solution, the edges of assembled gel molds were sealed with acrylamide solution (5 ml) polymerized for this purpose with 130 µl of 10% ammonium persulphate (APS) [BRL] and 13 ml of N,N,N',N' - tetramethylethylenediamine (TEMED) [BRL].

Table 2.6.2.8. Volumes of reagents used to cast nondenaturing PAGE gels. [a]Stock solution was prepared by dissolving 29 g of acrylamide [BRL] and 1 g of N,N'-methylenebisacrylamide [BRL] in 100 ml of water. [b]Solution was prepared freshly by dissolving 1 g of Ammonium persulfate [BRL] in 10 ml of water.

30% Acrylamide[a]	15.6 ml
10X TBE	6.0 ml
Water	37.6 ml
10% Ammonium persulfate[b]	0.42 ml
TEMED	42 µl
Total	60 ml

To the rest of acrylamide solution,42 µl of TEMED was added, mixed by swirling, and poured into the space between the two glass plates filling the space almost to the top. The appropriate comb was immediately inserted, and the solution was allow to polymerize for 60 minutes at room temperature. The bottom spacer was then removed and the gel attached to the electrophoresis tank. The reservoir was filled with the buffer (1X TBE), the comb carefully removed, and the wells immediately flushed out with the buffer. It is essential to do this as soon as the comb is removed, otherwise, small amounts of acrylamide solution trapped by the comb will polymerize in the wells, producing distorted bands of

DNA. DNA samples were mixed with appropriate amount of gel loading buffer and loaded into the wells. 6X gel-loading buffer was prepared as follows:

0.25% bromphenol blue [BDH]
0.25 xylene cyanol FF [Sigma]
30% glycerol [Sigma]
water to volume

As a DNA marker, 5 µl of ΦX174 Hae digest (see section 2.6.2.4.a) was run in parallel. The gel was run under the conditions given in Table 2.6.2.9.

Table 2.6.2.9. Electrophoresis conditions for nondenaturing PAGE gels

Gel type	Nondenaturing PAGE
Format	vertical
Run time	3-4 hrs
Gel concentration	8%
Gel lenght	20 cm
Gel thickness	1.5 mm
Gel buffer	1X TBE
Running buffer	1X TBE
Sample volume	20 µl
Voltage	4-5 V/cm
Temperature	ambient

The gel was run until the marker dyes have migrated the desired distance. The gel was then detached from the plates and stained with ethidium bromide (0.5 μg/ml) in 1X TBE for 30-45 minutes. Viewing and photographing was carried out on a UV light box.

2.6.4. Colony assay in semisolid media

In order to test for the capacity to grow in soft-agar, HTori3 and tumour cell lines were seeded in agar and in methyl cellulose.

2.6.4.1. Cloning in agar

The cell suspension was layered into 60 mm culture dishes [Nunc] containing a 2 ml underlayer of 0.9% (w/v) agar [Difco], penicillin 100 units/ml, streptomycin 100 μg/ml in Ham's F12/DMEM medium with no FCS. Triplicate dishes were inoculated with 10^2, 10^3 or 10^4 cells suspended in 2 ml of Ham's F12/DMEM medium containing 0.3% (w/v) agar,10% (v/v) FCS, penicillin 100 units/ml, streptomycin 100 μg/ml, and 2 mM L-glutamine. The culture were incubated for 21 days at 37°C in humidified air atmosphere with 5% CO_2.

The colonies were stained by adding 1 ml of staining solution per dish 12 hours before counting. The staining solution was prepared by dissolving 100 μg of 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyl-tetrazolium chloride (INT) [BDH], and 900 mg NaCl in distilled water (to 100 ml). After adding staining solution, incubation of the cultures were continued for 12 hours in 5% CO_2 fully humidified atmosphere at 37°C. As viable cells proliferate *in vitro* they convert the colourless

tetrazolium salt to a water insoluble red formazan which precipitates inside the cells (Bol *et al.*, 1977). Colonies with more than 50 cells were scored by microscopic examination.

2.6.4.2. Cloning in methyl cellulose

Methocel suspension (2% stock suspension) was first prepared. Methocel powder [Fluka] was autoclaved in a beaker and boiled distilled water poured into the beaker. The mixture was stirred continously on a magnetic stirer [Gallenkamp]. When the solution had cooled to approximately 50°C, double strenght RPMI 1640 medium [ICN Flow] was added and then the resulting mixture with continously stirred overnight at 4°C. The suspension was then aliquoted into plastic tubes, covered with tin foil, and stored at -20°C.

Cloning was performed in 1.2% Methocel suspension over an agar base. The cell suspension was layered into 60 mm culture dishes [Nunc] containing a 2 ml underlayer of 0.9% (w/v) agar in RPMI 1640 medium containing penicillin 100 units/ml and streptomycin 100 µg/ml. The cells were diluted (10^2, 10^3 or 10^4) with Methocel suspension containing 10% (v/v) FCS, penicillin (100 units/ml), streptomycin (100 µg/ml), and 2 mM L-glutamine. Experiments were carried out in triplicates. The cultures were incubated for 4 weeks at 37°C in humidified atmosphere containing 5% CO_2. Fresh Methocel suspension (1 ml per dish) with the same content was added every week. Following this 4 week incubation the number of colonies with more than 50 cells were scored by microscopic examination.

2.6.5. Growth curves of HTori3 cells and tumour cell lines on plastic

In order to compare growth rate on plastic between parent HTori3 cells and HT3x2γ (cell line established from radiation-induced tumour), cells were seeded into T-25 culture flasks (Nunc). Cells were trypsinized, and a single cell suspension was prepared by gently pipetting up and down the cell suspension using a 10 ml pipette. After the cell count was determined, 2 x 10^4 cells in 5 ml of medium containing 7% FCS were seeded into T-25 flasks. The cultures were gassed with CO_2 and incubated at 37°C, with a medium change every third day.

Cell counts were taken daily using a Coulter counter (see section 2.6.1.1.). Experiments were carried out in triplicate. Mean values of cell numbers per flask were plotted against time in days.

2.6.6. Scanning electron microscopy (SEM)

The cells were grown in culture flasks to various cell densities. The medium was removed and the cells were rinsed in PBS. The cultures were fixed using glutaraldehyde/paraformaldehyde fixative for 1 hour and rinsed in the buffer. A disc of plastic was punched out with a heated copper tube cutter. The samples were dehydrated through ascending percentages of ethanol (50%; 70%; 90%; 100%) and stored in 70% alcohol until the final critical point drying and gold coating.

Ethanol was replaced with liquid carbon dioxide at the CO_2 tank pressure in a critical point dryer (SEMIDRI-780). When replacement was completed the CO_2 is then raised past its critical point by heating the CO_2.

The pressure is then allowed to drop slowly to room temperature. The specimen is now completely dry.

The specimens were then attached to aluminium stubs with double sided sticky tape and coated with a layer of gold using a sputter coater (EMSCOPE SC500) for 2 minutes at 15 mA. Observation was carried out under electron beam (JEOL 35CF) at low kilovoltage (10 kV).

Chapter 3

RESULTS

3.1. Transformation of HTori3 cells by γ-irradiation

3.1.1. Transformation of HTori3 cells following single doses of γ-irradiation

3.1.1.1. Experimental procedure

Exponentially growing HTori3 cells in culture flasks were exposed to single doses of 0, 0.5, 1, 2, 3, and 4 Gy of ^{137}Cs γ-irradiation. Following irradiation, the cells were subcultured for a period of 6 weeks. After each subculture the cells were allow to reach subconfluency. Tumorigenicity of the cells was then determined by assaying the frequency of tumours arising in athymic nude mice after subcutaneous inoculation of ~$3x10^6$ cells. The mice were monitored for a period of 4-6 months as described in section 2.3.3. Un-irradiated HTori3 cells, but otherwise treated in the same way as irradiated ones, were used as a control.

3.1.1.2. Results

Tumours were observed in all the groups exposed to single doses of γ-rays (Figure 3.1.1.1.). Three tumours out of the 57 recipients which recieved the control non-irradiated cells were also observed. The data was analyzed using a G-test (Sokal & Rohlf, 1981). A comparison of the proportion of tumours in the irradiated groups with those in the control

suggests that there is a significant difference between the number of tumours produced following irradiation as compared to the untreated control (G = 36.3; degrees of freedom (df) = 1; $p<0.001$). A similar comparison between, for example, the group receiving the cells irradiated with 2 Gy with the control group, suggests a significant difference in the proportions with tumours (G = 14.2; df = 1; $p<0.001$).

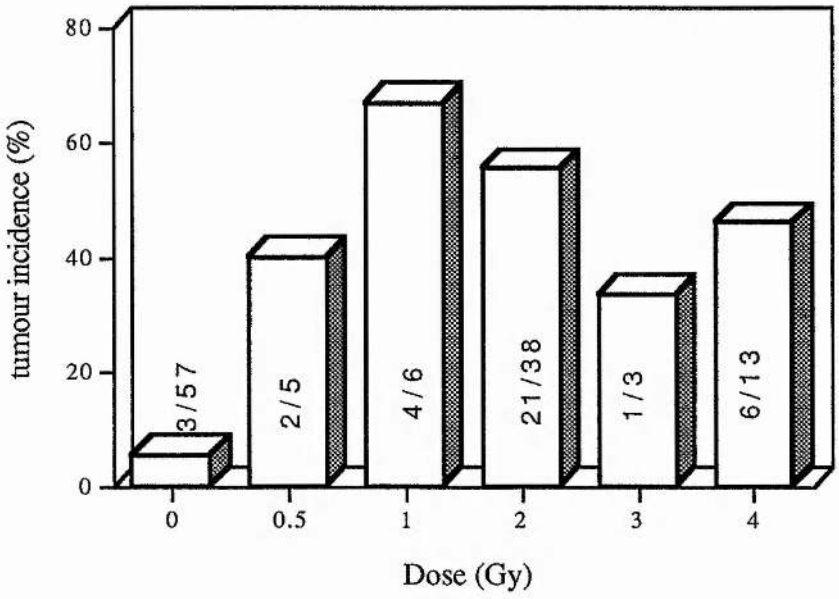


Figure 3.1.1.1. Tumour incidence in athymic nude mice following injection of HTori3 cells exposed to different single doses of γ-irradiation. Figures represent the number of tumours observed in the number of athymic nude mice injected.

There appears to be a trend, with increasing doses of radiation, tumour incidence increases, which then reaches a plateau, following which tumour incidence decreases. However, when the irradiated groups were compared to one another using the G-test for homogeneity the values of G_H were clearly not significant. It can be concluded that there is no evidence to suggest that there is any significant heterogeneity between these groups.

3.1.2. Transformation of HTori3 cells following multiple doses of γ-irradiation

3.1.2.1. Experimental procedure

HTori3 cells growing in culture flasks were exposed either to 3 or 6 doses of 0, 0.5, 1, 2, 3, and 4 Gy of ^{137}Cs γ-irradiation. Between individual exposures cells were passaged once and allowed to reach subconfluence before next exposure. Following final irradiation, the cells were subcultured for a period of 6 weeks, at which time they were assayed for tumorigenicity using a technique employing athymic nude mice as described in section 3.1.1.1.

3.1.2.2. Results

Figure 3.1.2.1. shows the tumour incidence in nude mice injected with the cells which were irradiated with multiple doses of γ-radiation. In the groups receiving 3 equal fractions of γ-rays, tumours were observed in the 1 Gy, 2 Gy and 3 Gy groups, with a relatively small number in the controls and the 0.5 Gy group. No tumours were observed in the group receiving cells exposed to multiple doses of 4 Gy. Statistics suggest that there is a significant difference between the number of tumours in groups receiving irradiated cells as compared to the unirradiated control ($G = 13.3$; $df = 1$; $p<0.001$) and also in the group receiving cells irradiated with 2 Gy as compared to the controls ($G = 14.6$; $df = 1$; $p<0.001$).

In the experiments involving multiple dose of γ-radiation, there appears to be a dose-effect relationship which can be described graphically by a bell shaped curve. However, the values of G_H obtained following a G-test to test for homogeneity were not shown to be significantly different

between the irradiated groups, with the exception of the group receiving 3 fraction of 4 Gy (G_H = 26.5; df = 5; $p<0.001$). When the overall tumour incidence of single doses of γ-rays was compared with those of multiple doses of γ-rays, no significant difference was obtained (G = 0.28; df = 1; $p<0.5$).

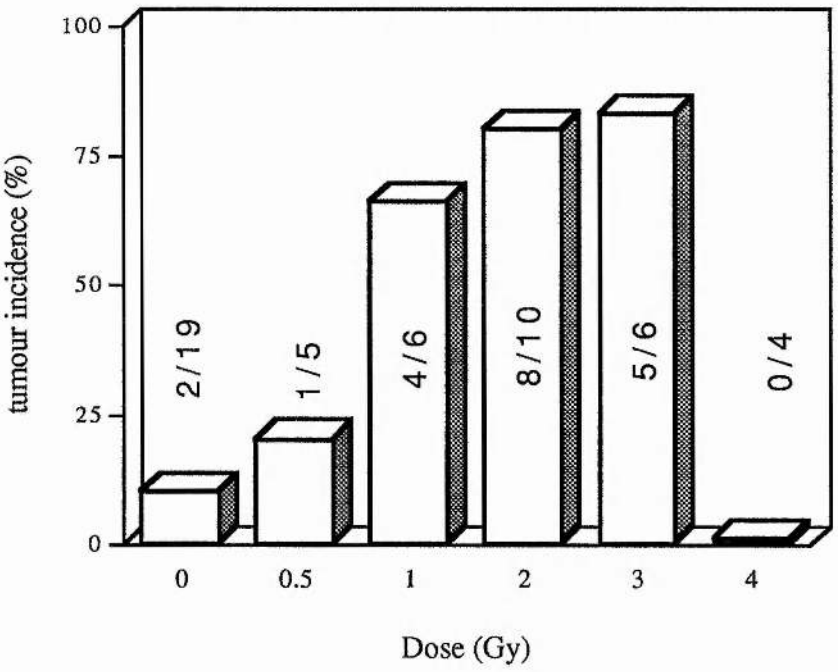


Figure 3.1.2.1. Tumour incidence in athymic nude mice following injection of HTori3 cells exposed to multiple (x3) doses of γ-irradiation. Figures represent the number of tumours observed in the number of athymic nude mice injected.

3.1.3. Latent period for primary tumours induced by γ-irradiation

The latent period may be defined here as the time between the injection of the treated cells and the appearence of a palpable tumour (size of 2 mm in diameter). The pattern of appearance of tumours was similar in these two experiments, the earliest tumour having been

observed at between 40-50 days, with a spread of latent periods up to 160 days (Figure 3.1.3.1. and Figure 3.1.3.2.).

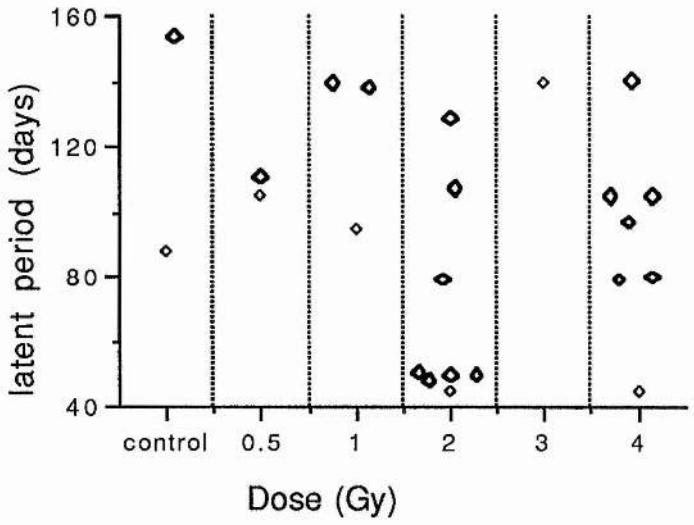


Figure 3.1.3.1. Latent period of primary tumours in athymic nude mice following injection of HTori3 cells irradiated with single doses of γ-irradiation.

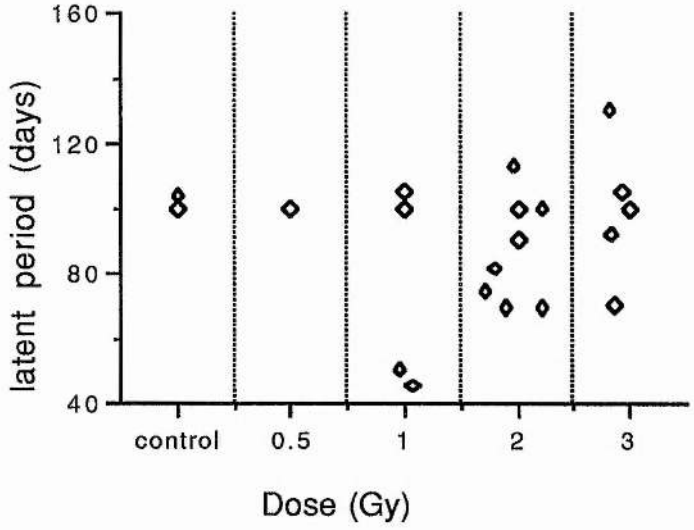


Figure 3.1.3.2. Latent period of primary tumours in athymic nude mice following injection of HTori3 cells irradiated with multiple doses of γ-irradiation.

3.1.4. Growth curves of primary tumours induced by γ-irradiation

The growth rates of primary tumours induced by single doses of γ-irradiation were measured and plotted as a function of time (Figure 3.1.4.1.). There was a relatively small differences in the growth rates of the individual primary tumours in the different treatment groups and also within the same treatment group. Similarly, there was a relatively small variations in the growth rate of primary tumours induced by multiple doses of γ-irradiation were observed (Figure 3.1.4.2.).

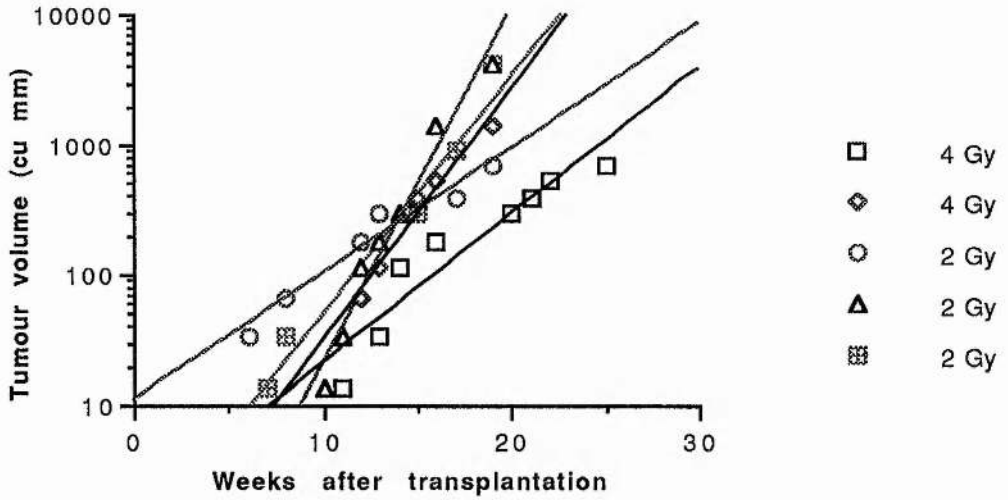


Figure 3.1.4.1. Growth rate curves of individual primary tumours in the athymic nude mice following injection of HTori3 cells irradiated with different single doses of γ-irradiation.

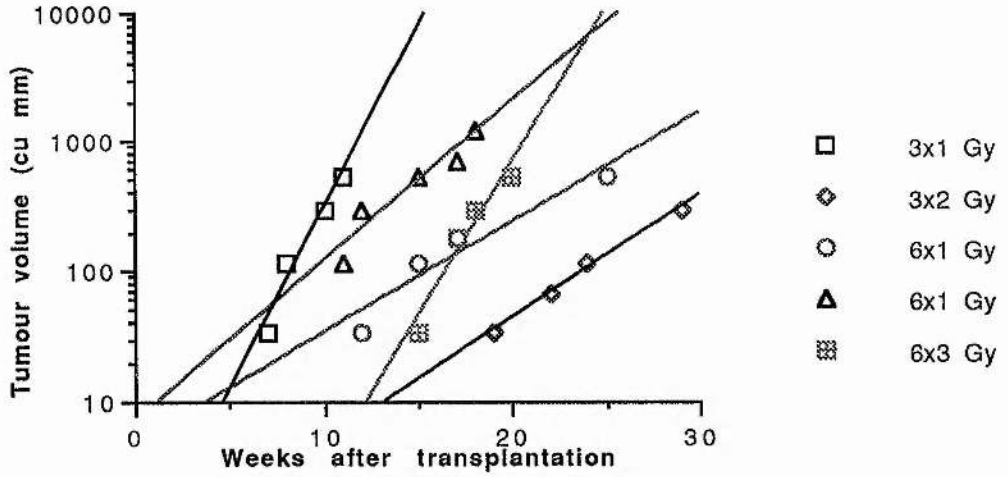


Figure 3.1.4.2. Growth rate curves of individual primary tumours in the athymic nude mice following injection of HTori3 cells exposed to multiple doses of γ-irradiation.

3.2. Transformation of HTori3 cells by α-particles

3.2.1. Tumour incidence following α-irradiation

3.2.1.1. Experimental procedure

HTori3 cells (2.5×10^5) were plated in specially designed dishes (see section 2.3.2.) 24 hours prior to irradiation to ensure that the cells were in exponential growth during the time of exposure. Cells were exposed to single doses of 0; 0.125; 0.25; 0.5; 1; and 1.5 Gy of ^{238}Pu α-particles. Following irradiation, the cells were removed from the dishes by routine trypsinization and plated into T-75 culture flasks. The cells were subcultured for a period of 6 weeks, at which time they were assayed for tumorigenicity (see section 2.3.3.).

3.2.1.2. Results

Figure 3.2.1.1. shows the data for transformation of HTori3 cells following the exposure to single doses of α-particles. The transformation incidence was plotted as a function of dose. For all doses of α-radiation, neoplastic transformation was induced. No clear dose-tumour incidence dependency was observed.

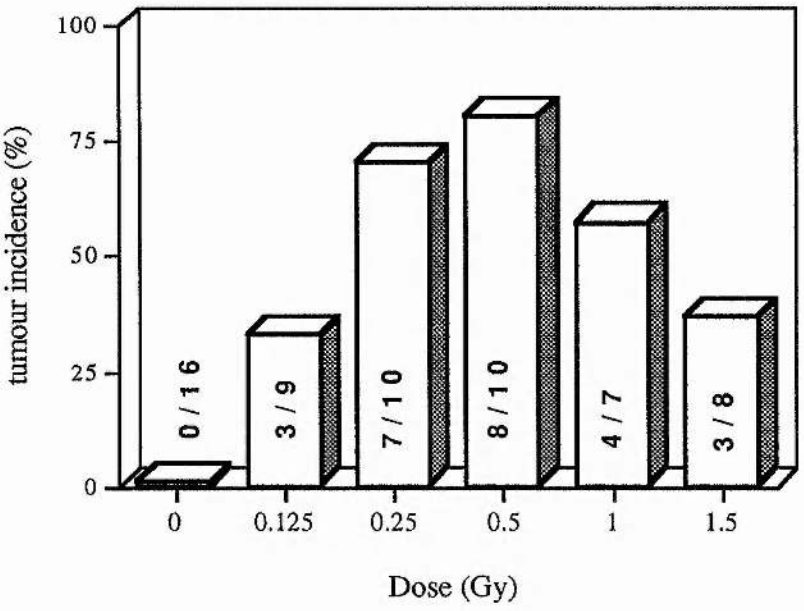


Figure 3.2.1.1. Tumour incidence in athymic nude mice following injection of HTori3 cells exposed to different single doses of α-irradiation. Figures represent the number of tumours observed in the number of athymic nude mice injected.

The G-test analysis suggested a significant difference between the control which recieved unirradiated cells as compared to the pooled number of tumours in groups recieving the irradiated cells ($G = 12.6$; $df = 1$; $p<0.001$) and compared to group receiving cells irradiated with 0.5 Gy ($G = 22.1$; $df = 1$; $p<0.001$).

In order to test whether the irradiated groups differ significantly from each other a G-test of the homogeneity was performed. The values

of G_H obtained were not shown to be significantly different suggesting that differences in the tumour frequencies between the different irradiated groups were non-significant.

Analysis of the overall tumour incidence with single doses α-particles as compared to single doses of γ-rays suggested that there was no significant difference between the ability of these two types of radiation treatment to induce neoplastic transformation ($G = 3.5$; $df = 1$; $p<0.1$). Similar to γ-radiation, there appears to be a trend, with increasing doses of α-radiation, the tumour incidence increases, reaches a maximum, following which tumour incidence decreases.

The Relative Biological Effectiveness (RBE) was calculated from the tumour incidence data as the ratio of γ-/α- doses to produce the maximum transformation incidence. The RBE of α-particles relative to γ-rays was about 4.

3.2.2. Latent period for primary tumours induced by α-irradiation

Similar to the γ-radiation data, most tumours observed following injection of α-treated cells had latent periods around 80-100 days (Figure 3.2.2.1.)

3.2.3. Growth curves of primary tumours induced by α-irradiation

Growth rate curves of primary tumours were also measured and plotted as a function of time (Figure 3.2.3.1.). There were small variations in growth rates of primary tumours observed after injection by the cells treated with different doses of α-particles.

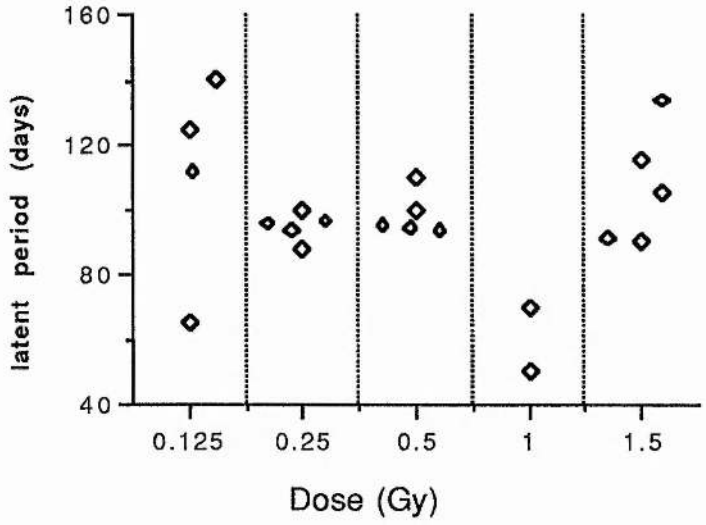


Figure 3.2.2.1. Latent period of primary tumours in athymic nude mice following injection of HTori3 cells irradiated with single doses of α-irradiation.

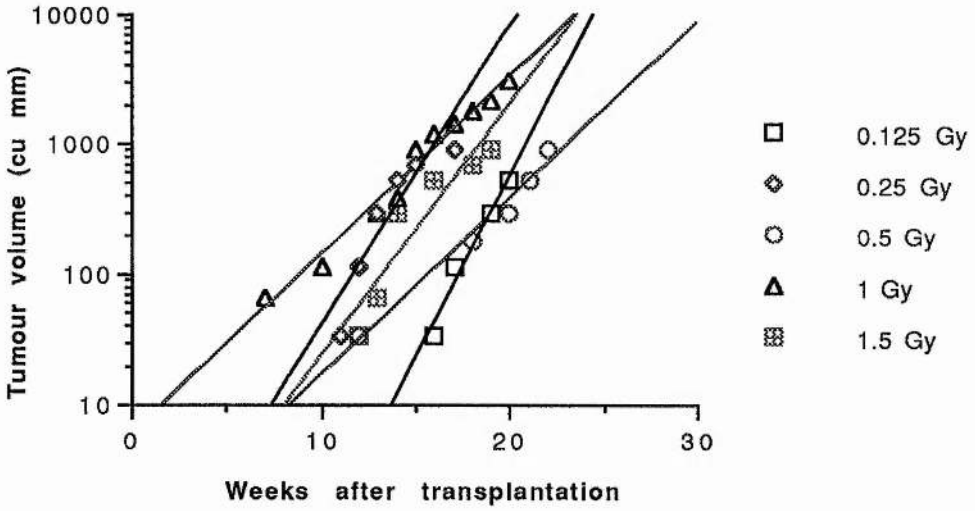


Figure 3.2.3.1. Growth rate curves of individual primary tumours in the athymic nude mice following injection of HTori3 cells irradiated with different single doses of α-irradiation.

3.3. Investigation of expression time in HTori3 cells following γ-irradiation

3.3.1. Experimental procedure

The effect of number of passages of HTori3 cells after γ-irradiation on tumour incidence in nude mice was investigated. Experimental protocol was schematically shown in Figure 2.3.1.1. The cells growing in

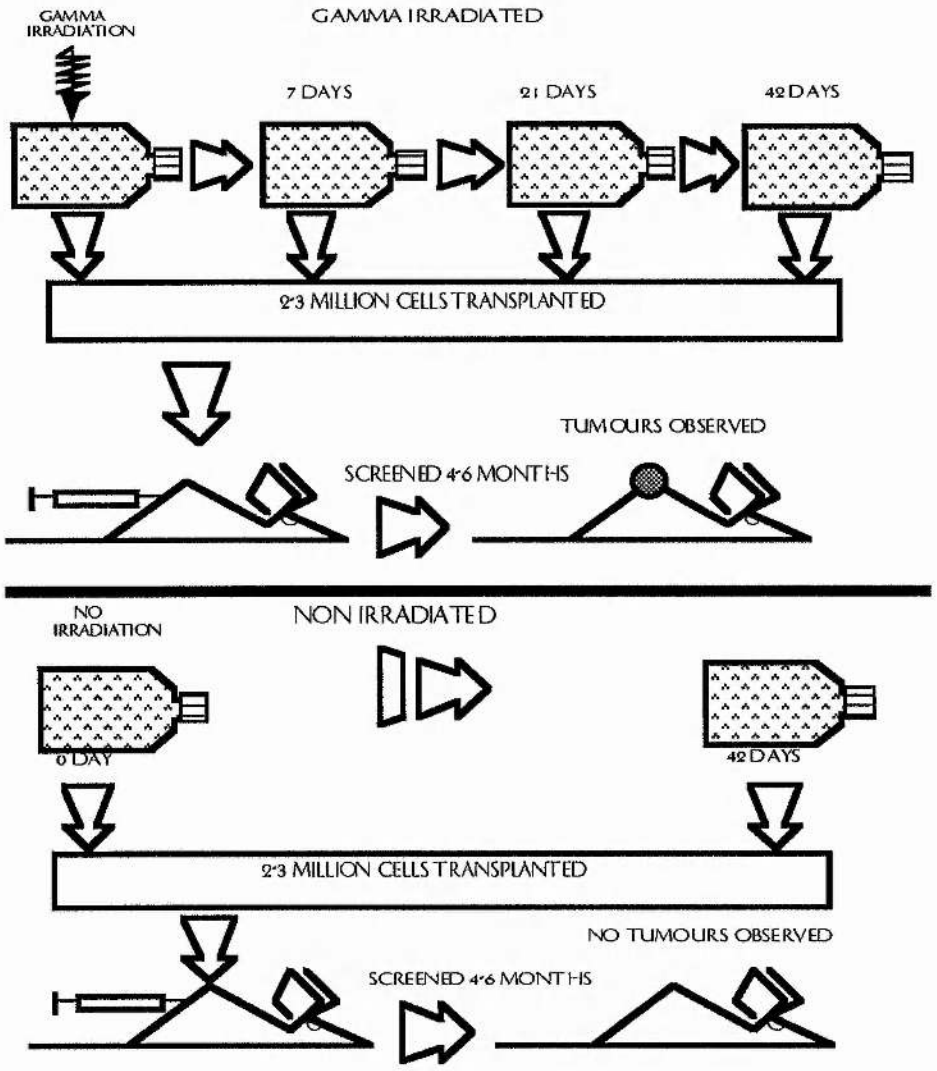


Figure 2.3.1.1. Schematic diagram shows experimental protocol used for investigation of expression time of HTori3 cells following γ-irradiation.

culture flasks were irradiated with 2 Gy of γ-irradiation. 2-3 x10^6 of the irradiated cells were transplanted into athymic nude mice (as described in section 2.3.3.) at 0, 7, 21, and 42 day after irradiation. Between irradiation and transplantation cells were passaged routinely once a week with medium change every 2 - 3 day (see section 2.1.1.). The animals were screened for tumour formation weekly for 3-6 months. Only progressively growing tumours were taken into account.

3.3.2. Results

Tumours were detected following transplantations of the irradiated cells at all time points tested (Table 3.3.1.1.). As a control un-irradiated HTori3 cells were transplanted into nude mice. No tumours were observed in the control groups.

Table 3.3.1.1. Tumorigenicity of radiation-treated HTori3 cells transplanted into athymic nude mice at 0, 7, 21, and 42 days after γ-irradiation. [a]Mice were injected with 2-3 x 10^6 viable cells. [b]Figures indicate the number of tumours observed in the number of recipients transplanted.

Time between irradiation and transplantation	Un-irradiated[a]	2 Gy γ-irradiation[a]
0 day	0/9[b]	4/5[b]
7 days	-	3/6
21 days	-	2/6
42 days	0/8	3/4

3.4. Histological examination of the primary tumours

When the tumours had attained a size of 5-9 mm in diameter, the animals were sacrificed and the tumours were bissected into two equal parts (Figure 3.4.1.). One part was used for the derivation of the cell line and the other for histological examination. Routine Hematoxylin and Eosin staining of paraffin-embedded sections revealed that the tumours consisted predominantly of areas of poorly differentiated cells with the occasional evidence of small follicular areas. The histological appearance of the primary tumour (2 Gy multiple dose) is illustrated in Figure 3.4.2.A. (low magnification) and Figure 3.4.2.B. (higher magnification). Numerous mitotic figures were observed. Histologically, the tumours appearance was consistant with that of undifferentiated, anaplastic carcinomas.

3.5. Confirmation of identity of the tumour cell lines

3.5.1. Cytokeratin staining

Keratins are a group of water-insoluble cytoskeletal proteins which form filaments in all epithelia. The presence of human cytokeratins is strongly suggestive of the human epithelial nature of the cells in question. Using immunocytochemical techniques (murine anti-cytokeratin antibody MNF 116) for staining human cytokeratins, the tumour cells were stained positively (Figure 3.5.1.1.) establishing the human epithelial nature of the cell lines derived from tumours grown in nude mice.

3.5.2. SV40 large T-antigen detection

Using the monoclonal antibody PAb 405, SV40 large T-antigen was detected in the nuclei of the tumour cell lines established (an example shown in Figure 3.5.2.1.). The presence of this specific antigen in the nuclei shows that SV40 large T-antigen is present in these cells.

3.5.3. Chromosome analysis

Chromosome analyses was carried out in order to confirm the human origin of the tumour cell lines. Following the initial explantation, a mixture of both human and mouse karyotypes were present, with human types predominating. After 2 - 3 subsequent passages only human karyotypes were observed.

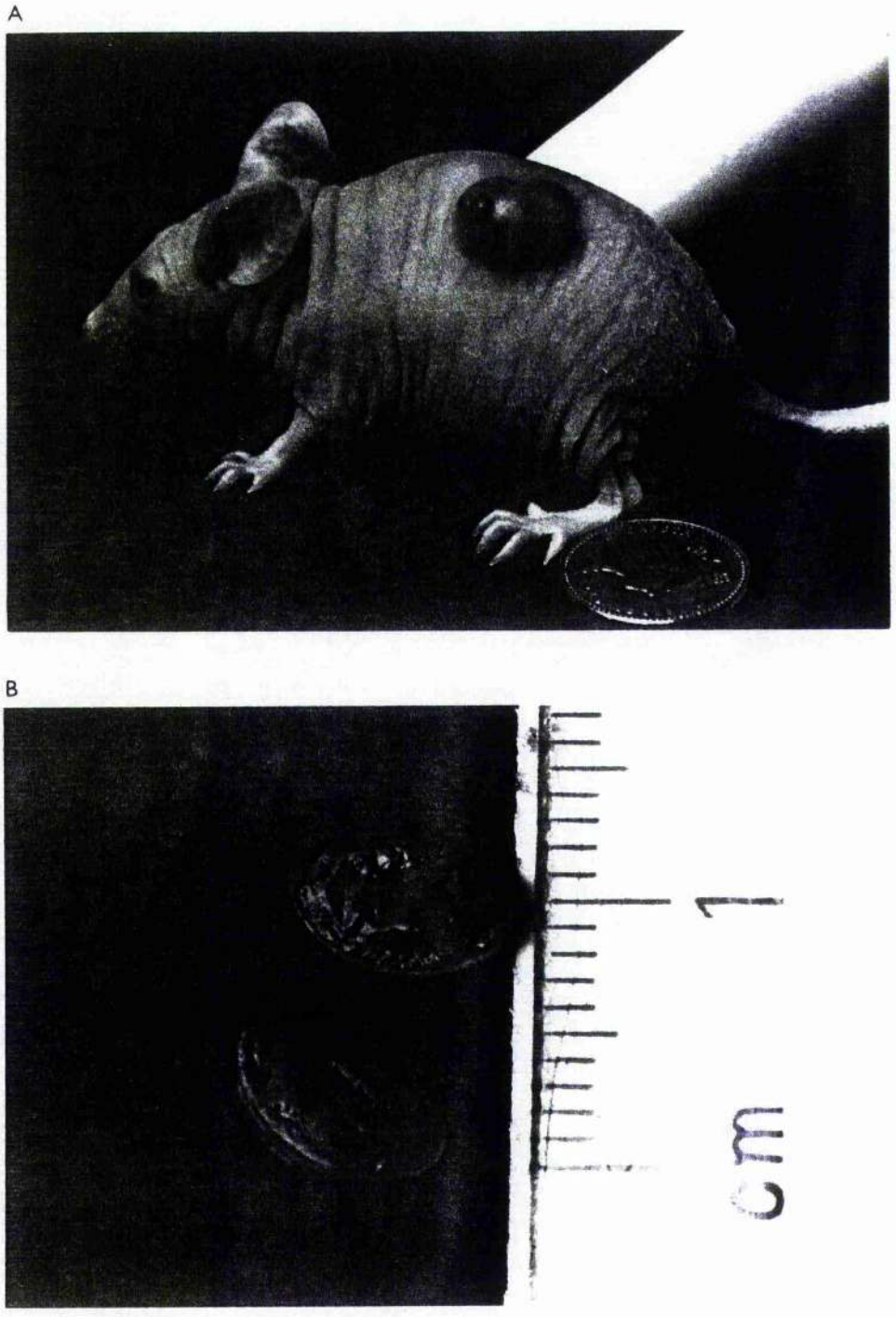


Figure 3.4.1. Examples of tumour growing in athymic nude mouse.following s.c. injection of 2-5 x 10^6 cells. A: a primary tumour generated after radiation treatment (3 doses of 2 Gy γ-irradiation) of HTori3 cells. The site of tumour formation corresponds to the the site of injection. B: appearance of a primary tumour at cross section. Note a solid nature of the tumour without any sigh of necrosis.

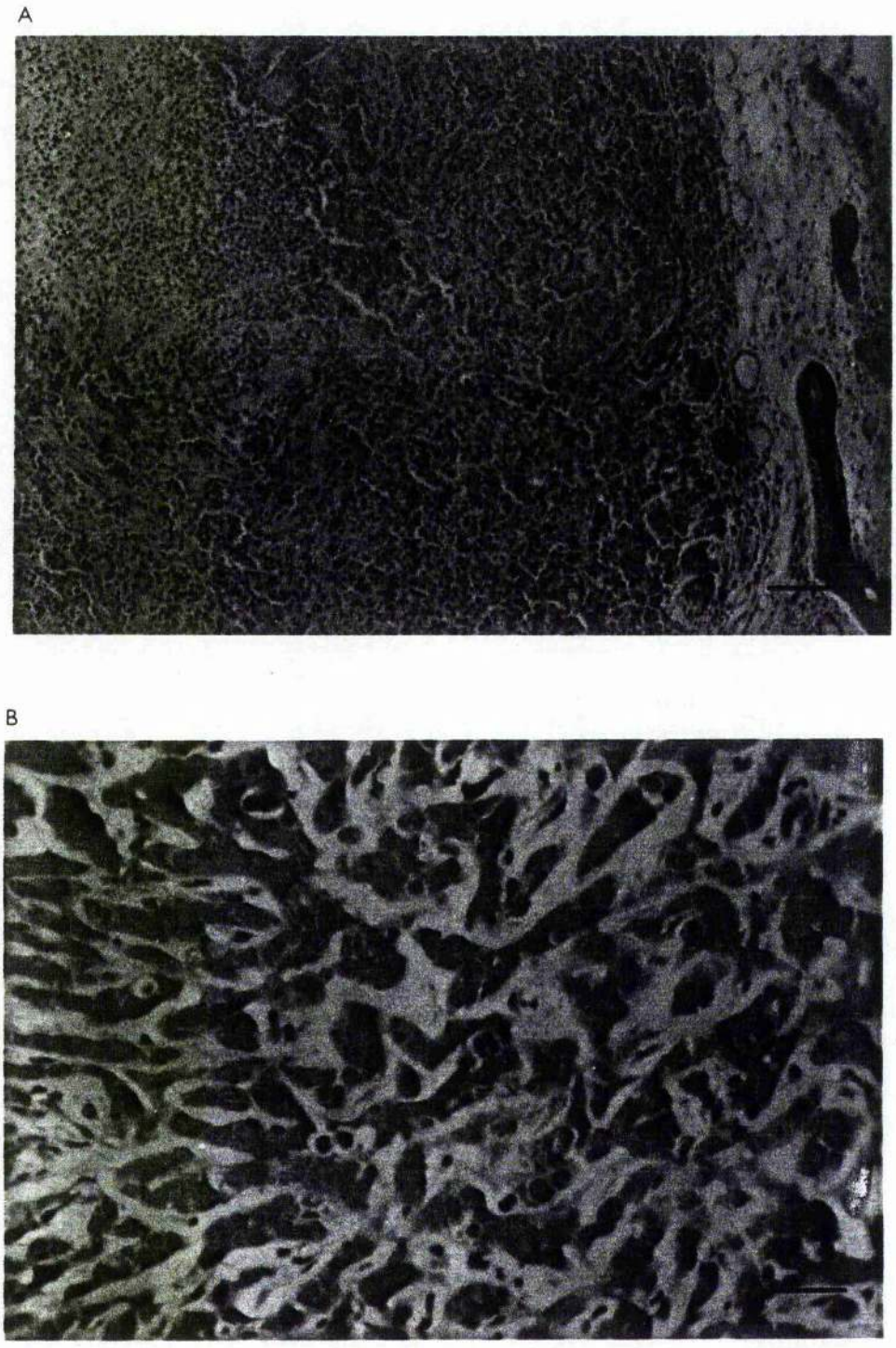


Figure 3.4.2. Histopathological section of a primary tumour grown in athymic nude mice following s.c. inoculation of HTori3 cells irradiated with 1 Gy γ-radiation. A: the section shows a poorly defferentiated primary tumour (scale bar = 100 μm); B: the same section as A (scale bar = 25 μm). Paraffin sections were stained with Hematoxylin and Eosin.

Figure 3.5.3.1. shows one of the human karyotypes. Most karyotypes examined (20 metaphases) revealed chromosomal abnormalities, both numerical and structural, but the human origin was nevertheless ascertained.

3.5.4. DNA fingerprinting

To confirm that the tumour cell lines were derived from the parent cell line following radiation treatment, but not from other tumour cell lines used within the laboratory, DNA fingerprinting was performed. The DNA patterns were analysed following hybridization to RNA probes 19.6 (Figure 3.5.4.1.) or 18.15 (not shown) (Carter *et al.*, 1989). These probes are RNA analogues of multilocus 33.6 and 33.15 tandem-repetitive regions described by Jeffreys *et al.* (1985).

The DNA fingerprint of the parent cell line was compared with those of the tumour cell lines which were believed to be derived from it. Eleven resolvable bands were observed in parent line (lane a) all of them are shared with the all tumour cell lines obtained (lanes b - p). There were no bands in HTori3 cell line that were not shared with the tumour cell lines. A sample from other individual (T24 human bladder carcinoma cell line) were included for comparison (lane T) where no band sharing was observed.

From the comparison of DNA profiles of the control lane a with the tumour samples in lanes b - p it was confirmed that all the tumour cell lines were indeed derived from the same parent HTori3 cells. However, lane f showed novel bands (arrows) that could not be matched with the parent HTori3 cell line (lane a).

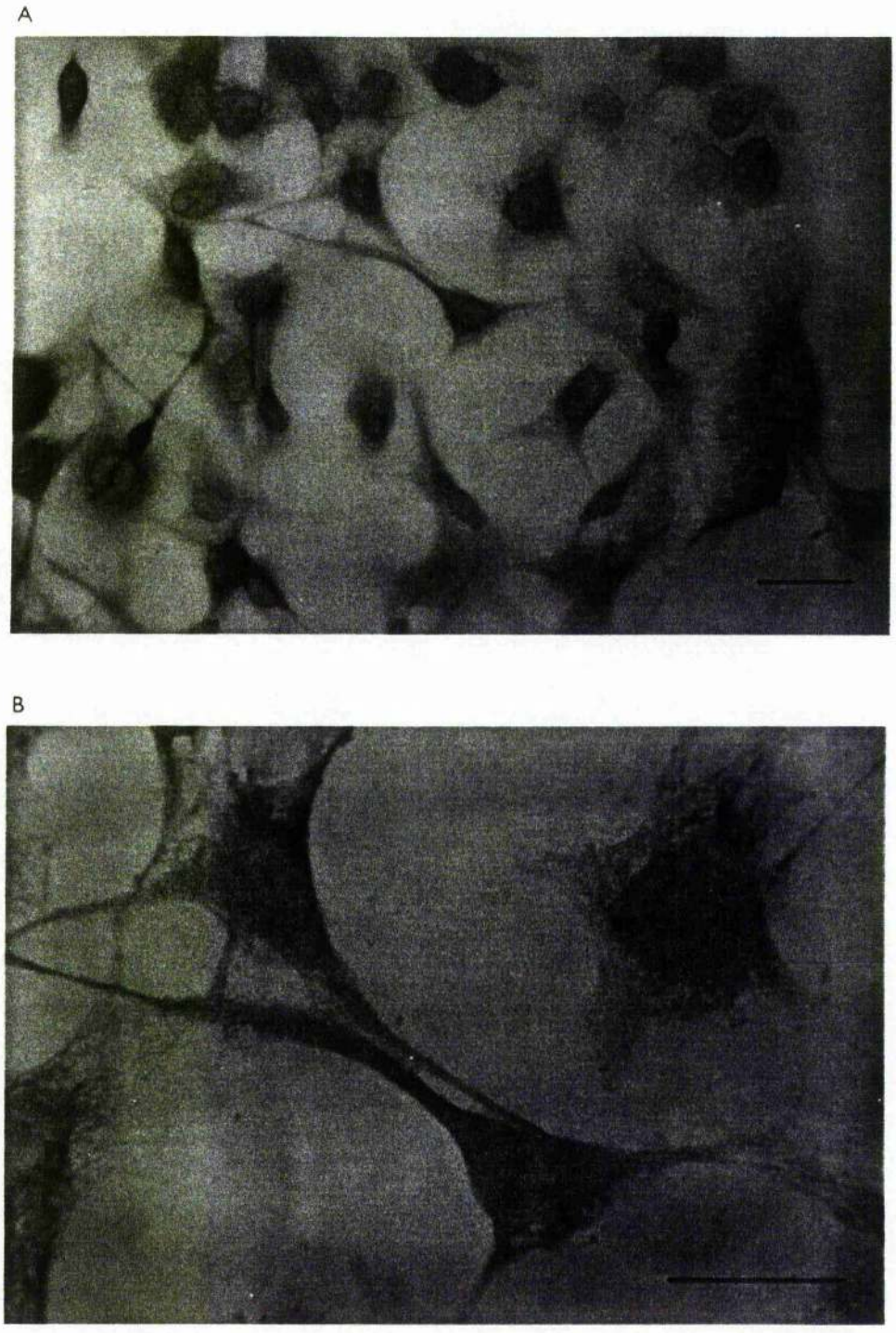


Figure 3.5.1.1. Immunocytochemical staining of human cyrokeratin in (A) parent HTori3 cell line (scale bar = 25 μm) and (B) radiation-transformed tumour cell line HT3x1γ (scale bar = 25 μm) using monoclonal DAKO MNF 116 antibody. Note cytoplasmic dark brown staining representing human cytokeratin.

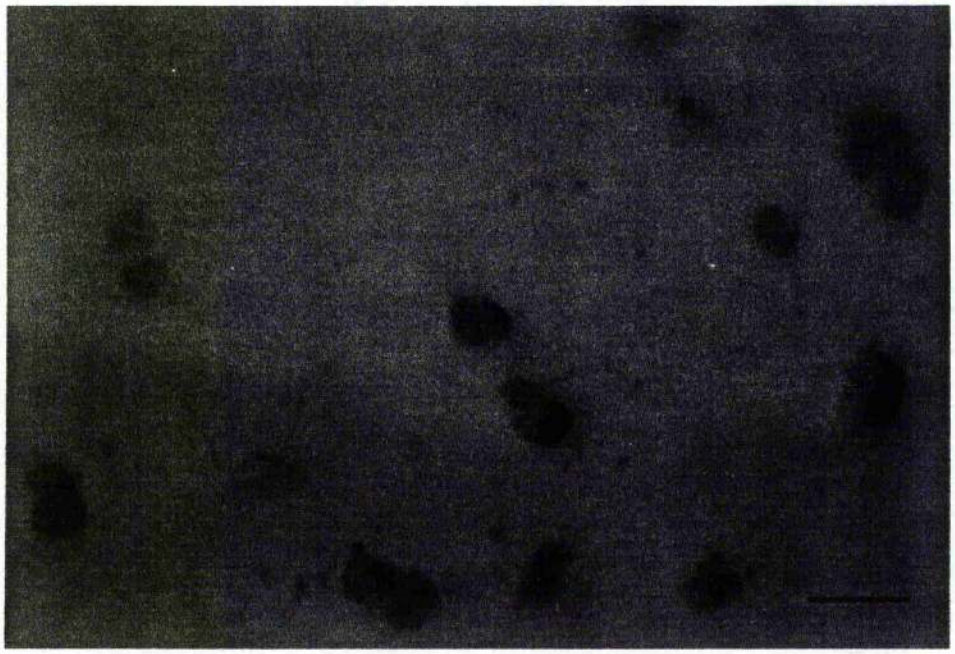


Figure 3.5.2.1. Immunocytochemical staining with monoclonal PAb 405 antibody against SV40 large T antigen in radiation-transformed tumour cell line HT3x2γ. Note nuclear brown staininghwhich represent the staining of the SV40 large T antigen (scale bar = 25 μm).

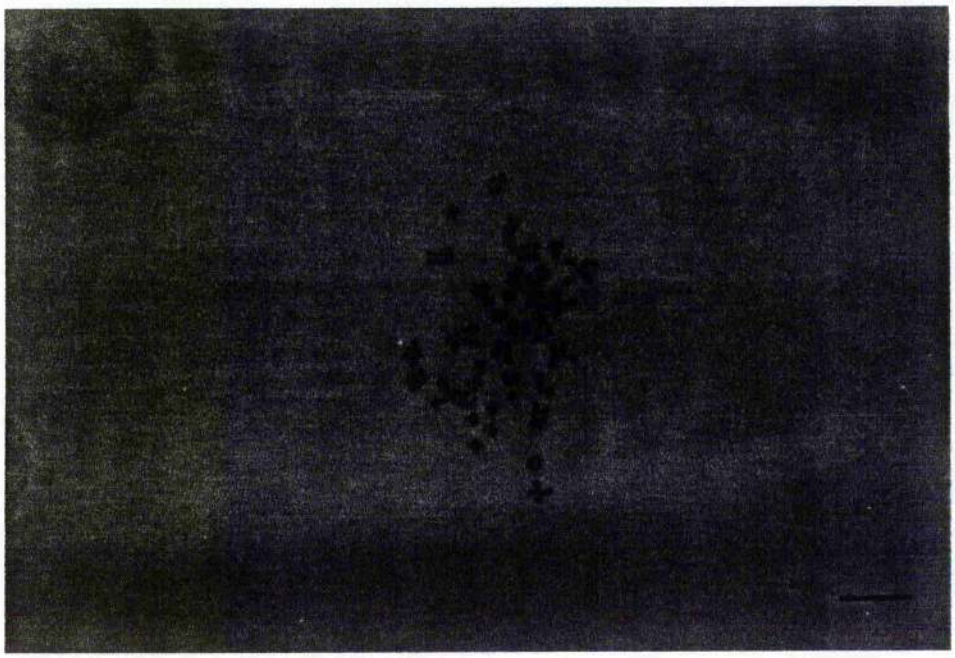


Figure 3.5.3.1. Microphotograph of chromosome spreads prepared from cell line established from a primary tumour grown in nude mouse after injection irradiated HTori3 cells (3 x 2 Gy γ-rays). Human karyotype confirms human origin of the cells (scale bar = 10 μm).

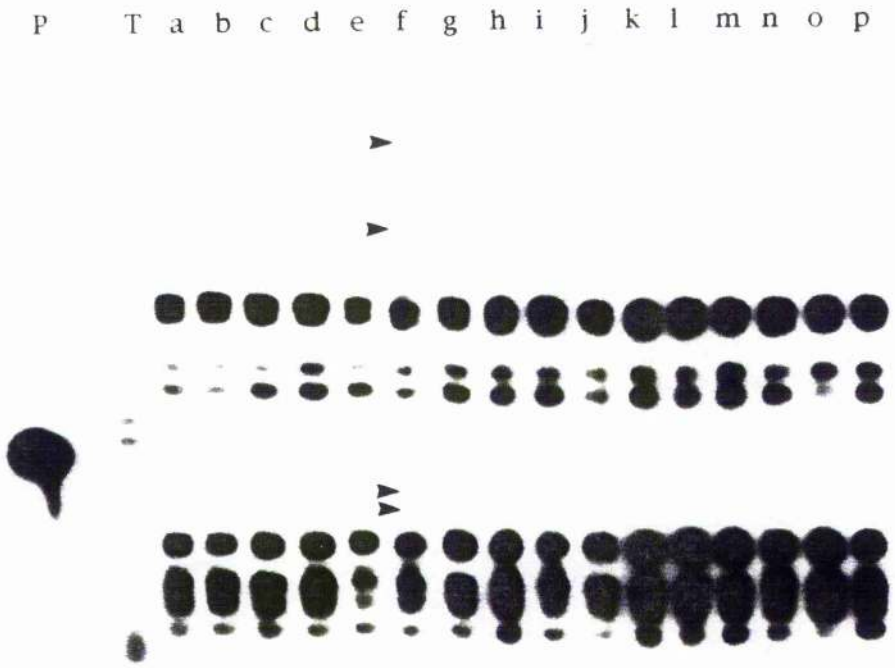


Figure 3.5.4.1. DNA fingerprints of the cell lines derived from tumour induced by ionizing radiation compared to presumptive parent HTori3 cell line. Autoradiograph of a Southern blot in which ^{32}P-labelled 19.6 RNA probe was used to probe Hae III digested DNA from individual cell lines. Lane P: plasmid DNA used as control for RNA probe; lane T: the T24 cell line used as a control sample from other individual for comparison purposes; lane a: parent HTori3 cell line; lanes b - p: the radiation-transformed tumour cell lines as follows: lane b: HT1x0.125α2; lane c: HT1x0.25α7; lane d: HT1x0.5α8; lane e: HT1x1α4; lane f: HT1x1.5α9; lane g: HT1x1γc; lane h: HT1x2γ7; lane i: HT1x4γ6; lane j: HT3x1γ; lane k: HT1x0.5α6; lane l: HT1x2γ9; lane m: HT1x0.5α1; lane n: HT1x0.5γT; lane o: HT3x3γ; lane p: HT1x0.25α4.

3.6. Characterization of radiation-transformed cell lines

The cell lines derived from primary tumours induced by γ- and α-irradiations are listed in Table 3.6.1. and Table 3.6.2.

3.6.1. Morphological characteristics

Morphology of cell culture of the parent HTori3 cell line and radiation-transformed tumour cell lines was examined by phase-contrast and scanning electron microscopy. Normal non-tumorigenic parent HTori3 cells are epithelial in morphology, with a tendency to grow as a closely packed uniform adherent monolayers in tissue culture (Figure 3.6.1.1.). Figure 1.6.1.1.B. shows typical appearance of HTori3 cells at confluence, a small number of mitotic figures are visible in a background of non-overlapping "cobblestone" like cells.

Cultures established from primary tumours, which were generated by irradiation retained their epithelioid character however were more rounded and thickened (Figure 3.6.1.2. and Figure 3.6.1.3.C. and D.). This is in contrasted with the thin, almost transparent configuration of the parent HTori3 cells (Figure 3.6.1.3.A. and B.).

The tumour cell lines were characterized by a heterogeneous morphological appearance and appeared to reach a higher saturation density as compared to the parent HTori3 cells. The increased frequency of round cells in the tumour cell cultures suggest an increased mitotic activity which continued even at confluence. The tumour cells were varied sizes and were irregulary shaped as compared to the parent cells.

Table 3.6.1. Tumour incidence of HTori3 cells following irradiation with single and multiple doses of γ-irradiation, cell line established and tumorigenicity of these cell lines. [a]Number of tumours scored per number of athymic nude mice injected, only progressively growing tumours following injection of 2 - 3 x 10^6 cells. [b]NT - not tested.

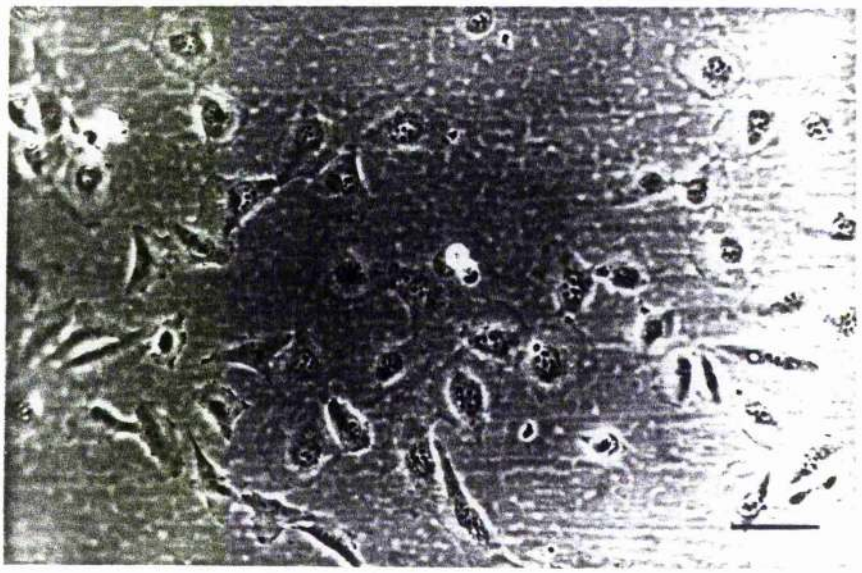
Dose (Gy x no.of exposure)	Tumour incidence[a]	Number of cell lines established	Cell line designation	Tumorige-nicity[a]
0.5 x 1	2/5	1	HT1x0.5γT	4/4
0.5 x 3	1/5	-	-	-
1 x 1	4/6	1	HT1x1γC	2/2
1 x 3	4/6	1	HT3x1γ	3/3
1 x 6	2/3	1	HT6x1γ	1/1
2 x 1	9/17	4	HT1x2γ1	NT[b]
			HT1x2γ6	2/2
			HT1x2γ7	4/4
			HT1x2γ9	4/4
2 x 3	8/10	2	HT3x2γ	6/6
			HT3x2γX	NT[b]
3 x 1	1/3	-	-	-
3 x 3	5/6	1	HT3x3γ	4/4
3 x 6	1/2	1	HT6x3γ	NT[b]
4 x 1	6/13	-	HT1x4γ6	4/4
4 x 3	0/4	-	-	-
4 x 6	0/2	-	-	-

Table 3.6.2. Tumour incidence of HTori3 cells following irradiation with α-irradiation, cell line established and tumorigenicity of these cell lines. [a]Number of tumours scored per number of athymic nude mice injected, only progressively growing tumours following injection of 2 - 3 x 10^6 cells. [b]NT - not tested.

Dose (Gy x no.of exposure)	Tumour incidence[a]	Number of cell lines established	Cell line designation	Tumorige-nicity[a]
0.125 x 1	3/9	1	HT1x0.125α2	7/7
0.25 x 1	7/10	2	HT1x0.25α4	3/3
			HT1x0.25α7	3/3
0.5 x 1	8/10	4	HT1x0.5α1	4/4
			HT1x0.5α2	NT[b]
			HT1x0.5α6	3/3
			HT1x0.5α8	6/6
1 x 1	4/7	1	HT1x1α4	4/4
1.5 x 1	3/8	1	HT1x1.5α9	NT[b]

Most tumour cell lines exhibited a random pattern which contrasted with the regular organized arrangements of HTori3 cells. No piling up of tumour cells was observed except one focus found in culture of the HT3x1γ cell line. Figure 3.6.1.3.E. and F. shows a scanning electron photomicrograph of a focus with massive piling up of round cells varying in size. Criss-crossing was not pronounced.

A



B

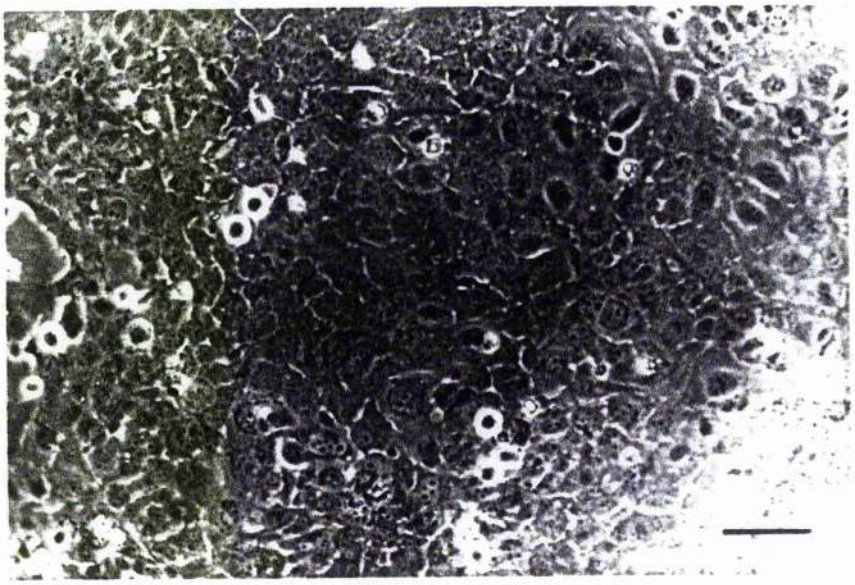
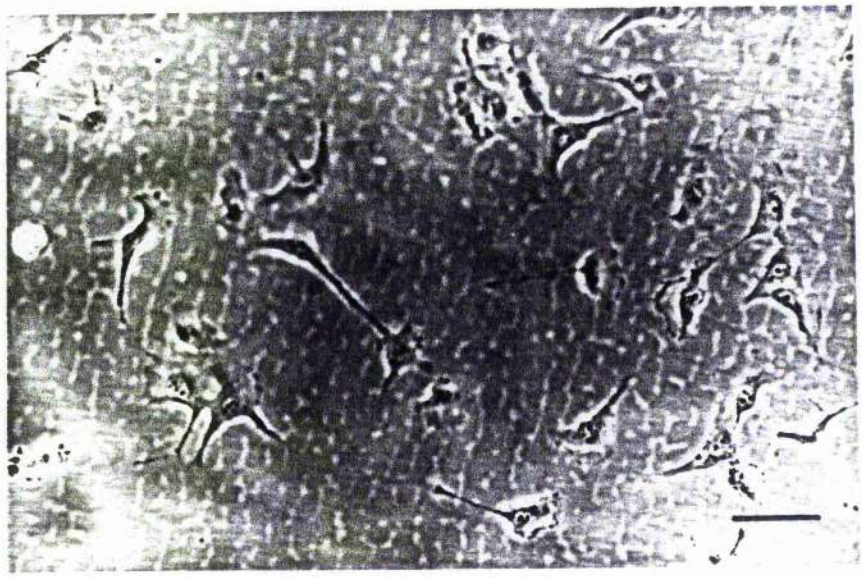


Figure 3.6.1.1. The morphology of the parent HTori3 cells. Phase-contrast photographs were taken of the cells growing as a monolayer in culture flasks. A: HTori3 cells at low density; B: HTori3 cells as confluent monolayer. Scale bar represents 25 μm.

A



B

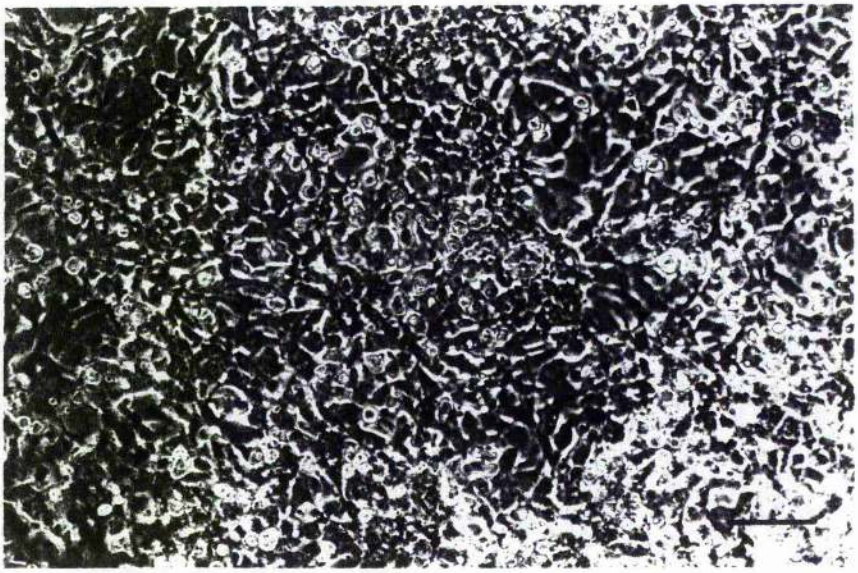
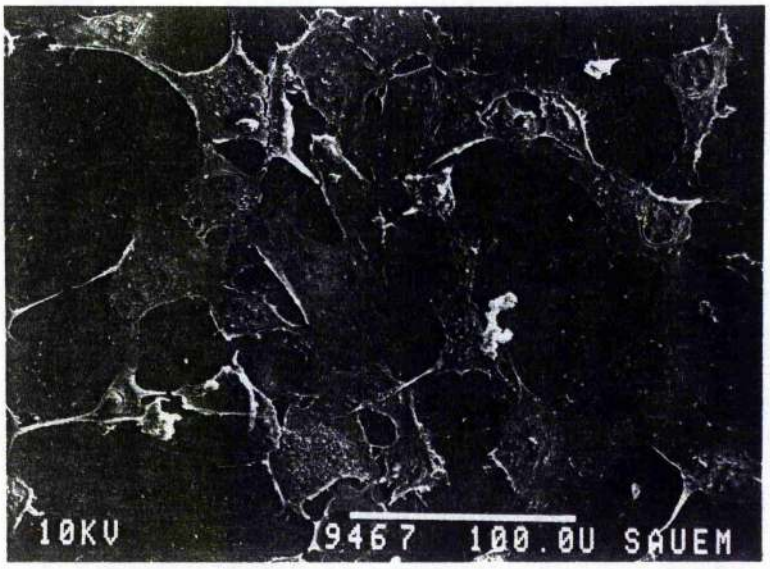


Figure 3.6.1.2. The morphology of the cell line derived from radiation-induced primary tumours. Phase-contrast photographs were taken of the cells growing as a monolayer in culture flasks. A: HT3x2γ cell line at low density; B: HT3x2γ cell line at confluence. Note more densely packed cells with numerous mitotic figures even at confluence. Scale bar represents 25 μm.

A



B

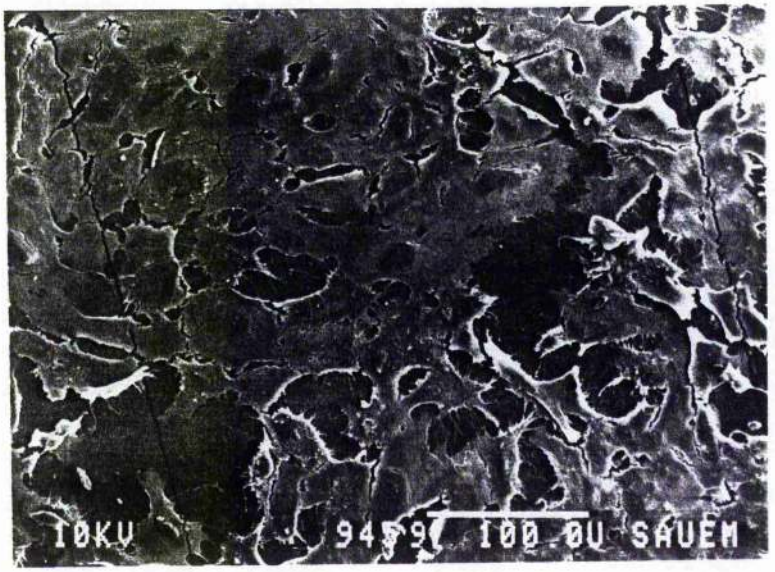
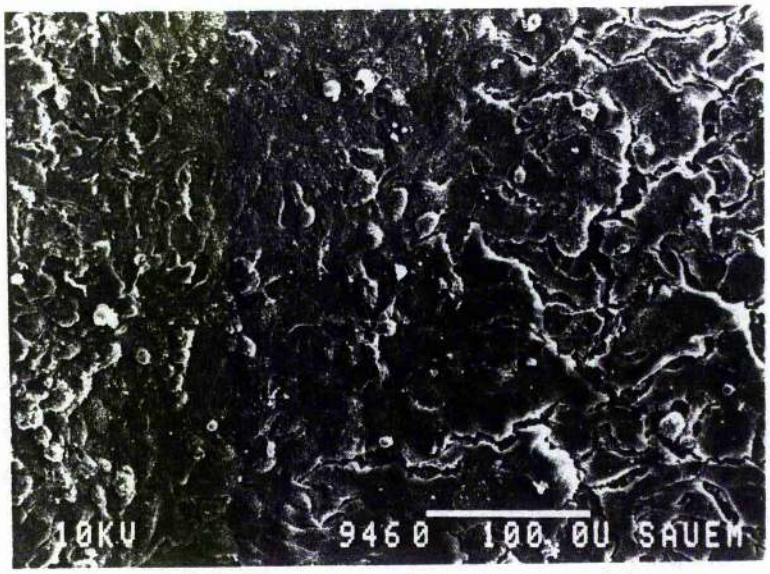


Figure 3.6.1.3. Scanning electron microphotographs of the parent HTori3 cells. A: HTori3 cells at low densities (scale bar = 100 μm); B: HTori3 cells at hight density (scale bar = 100 μm).

C



D

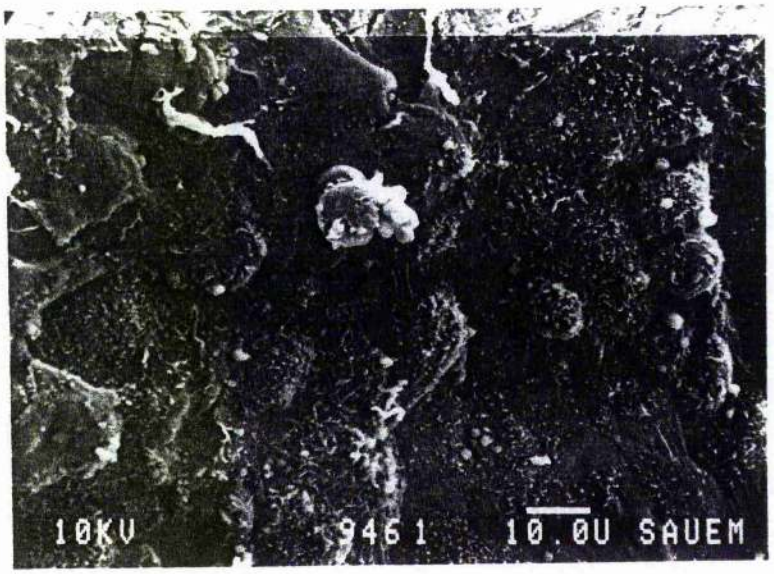
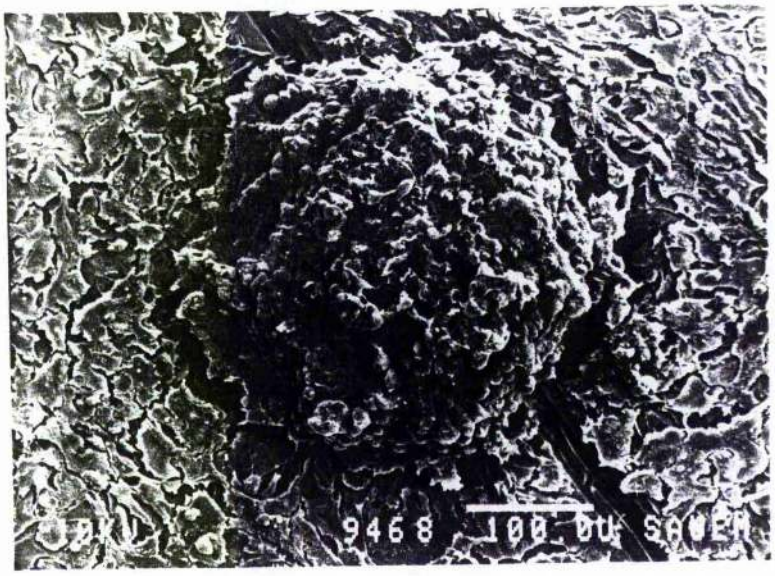


Figure 3.6.1.3. Scanning electron microphotographs of the radiation-derived tumour cell lines. C: HT1x0.25α4 cells (scale bar = 100 μm); D: HT1x0.25α4 at higher magnification (scale bar = 10 μm).

E



F

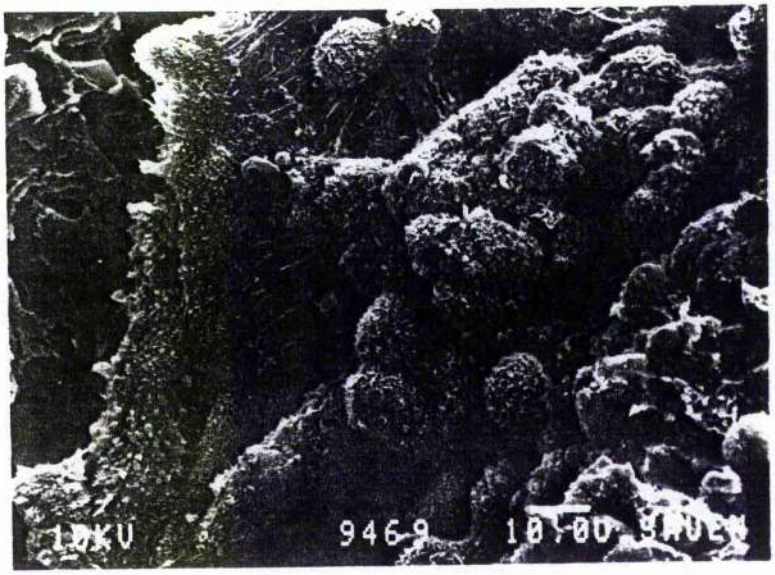


Figure 3.6.1.3. Scanning electron microphotographs of the radiation-derived tumour cell line. E: a focus found in culture of HT3x1γ cells (scale bar = 100 μm); F: a focus found in culture of HT3x1γ cells at higher magnification (scale bar = 10 μm).

3.6.2. Tumorigenicity of the tumour cell lines

The derived tumour cell lines were further inoculated into nude mice to test tumorigenicity. Results are shown in Table 3.6.1. and Table 3.6.2. All cell lines tested, resulted in the production of tumours upon injection of 2-3 x 10^6 cells in the nude mice. The latent periods of secondary tumours were on average shorter in comparison with latent periods of primary tumours. The earliest tumours were observed at between 10-15 days with a spread of latent periods up to 6 weeks.

3.6.3. Growth curves of secondary tumours

The growth rate of secondary tumours were also measured and the results are illustrated in Figure 2.6.3.1. and Figure 2.6.3.2. For the tumours

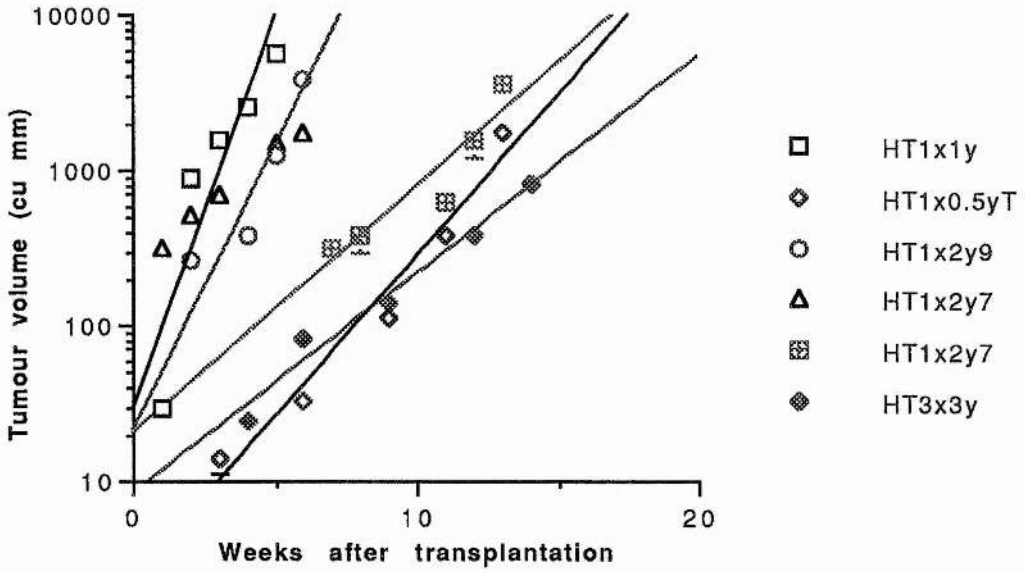


Figure 3.6.3.1. Growth rate curves of secondary tumours in the athymic nude mice injected with cell lines derived from primary tumours generated by γ-irradiation of HTori3 cells *in vitro*.

obtained after injection of cells from various tumour cell lines, there was a little variation in tumour growth rates.

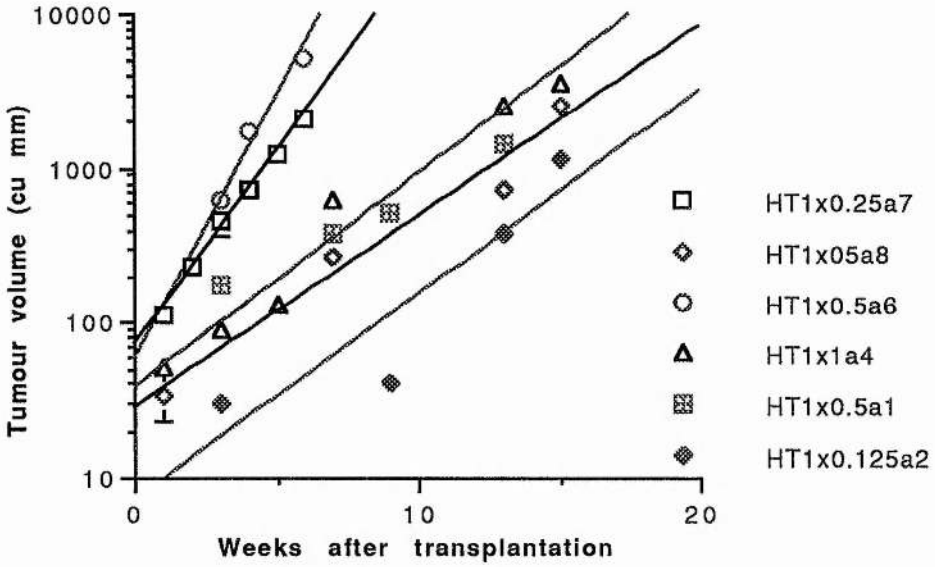


Figure 3.6.3.2. Growth rate curves of secondary tumours in the athymic nude mice injected with cell lines derived from primary tumours generated by α-irradiation of HTori3 cells *in vitro*.

3.6.4. Cell concentration and tumour growth rate

In order to investigate whether the cell concentration affected the tumour growth rate, nude mice were injected with varying cell concentration of two different cell lines: the HT3x2γ (Figure 3.6.9.1.) and the HT1x0.5α8 (data not shown). Cell concentrations ranged from 5×10^6 to 3×10^4 cells. Similar tumour doubling times and tumour growth rates were observed in animals receiving 5×10^6, 5×10^5 and 2×10^5, although the period of latency was longer when lower cell doses are injected. No progressive tumour growth was observed in animals receiving 3×10^4 cells.

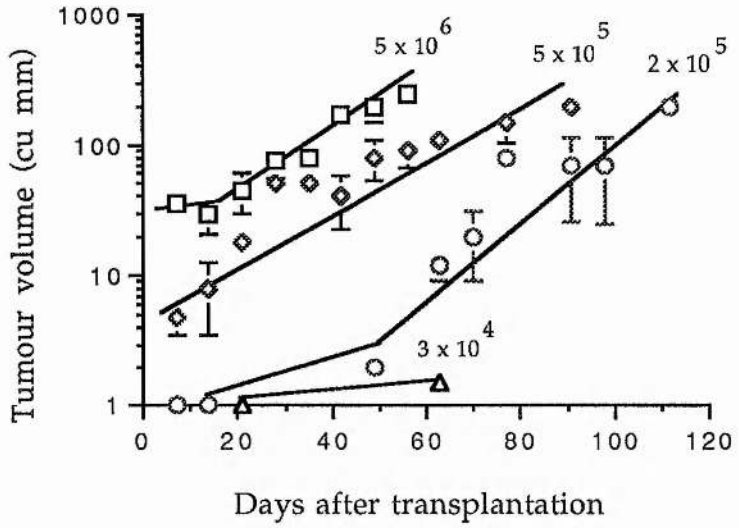


Figure 3.6.4.1. Growth curves of tumours in the nude mice receiving subcutaneous inoculations of different cells doses of a cell line derived from explants of a primary tumour produced by irradiating a human thyroid cell line in vitro (3 x 2 Gy γ-irradiation).

3.6.5. Plating efficiency and survival curves

The plating efficiency of HTori3 cells was calculated as the proportion of the cells capable of forming colonies when plated in tissue culture dishes. Plating efficiencies and surviving fractions of the HTori3 cell line and 6 tumour cell lines are shown in Table 3.6.5.1. For simplicity, when comparing the radiosensitivity of the tumour cell lines with parent HTori3 cells, only two dose points (2 Gy and 4 Gy) were used.

Figure 3.6.5.1. shows the survival data of HTori3 cells irradiated with graded doses of γ-rays and α-particles. The shape of the survival curve obtained with γ-rays, with an initial shoulder, is characteristic of that commonly observed for mammalian cells exposed to sparsely ionizing radiation. In this semi-logarithmic plot, at higher doses of γ-

Table 3.6.5.1. Plating efficiencies and cell survival of HTori3 cells and 6 tumour cell lines. Results are based on triplicate experiments unless otherwise stated. [a]Based on duplicate experiments; [b]Values are mean ± standard error of mean (S.E.M.). PE = plating efficiency.

		Surviving fraction	
Cell line	PE (%)[b]	2 Gy[b]	4 Gy[b]
HTori3	24.36±2.42	0.46±0.03	0.15±0.03
HT1x1γC[a]	15.2±4.25	0.36±0.02	0.09±0.01
HT1x2γ6	57.03±6.04	0.42±0.01	0.09±0.02
HT1x2γ7[a]	15.00±4.61	0.25±0.02	0.034±0.01
HT3x1γ	26.0±2.54	0.46±0.03	0.12±0.04
HT3x2γ[a]	16.50±2.66	0.11±0.01	0.02±0.005
HT3x3γ	18.0±1.77	0.36±0.03	0.1±0.02

radiation and all doses of α-particles, the survival curve can be described as a straight line. The D_o value was estimated to be 160 cGy and 70 cGy for γ- and α-irradiation, respectively.

The Relative Biological Effectiveness (RBE) of α-particles, calculated as the ratio of Do values for γ-radiation and α-particles, was 2.3. The RBE was also calculated from cell survival data at 10% survival level, and was estimated to be approximately 4.

In order to compare the survival of the parent HTori3 cells with that of the tumour cell lines, the survival curves for 6 tumour cell lines and the parent HTori3 cell line were plotted in the same graph (Figure 3.6.5.2.). A two way analysis of covariance (Ancova) showed that the line

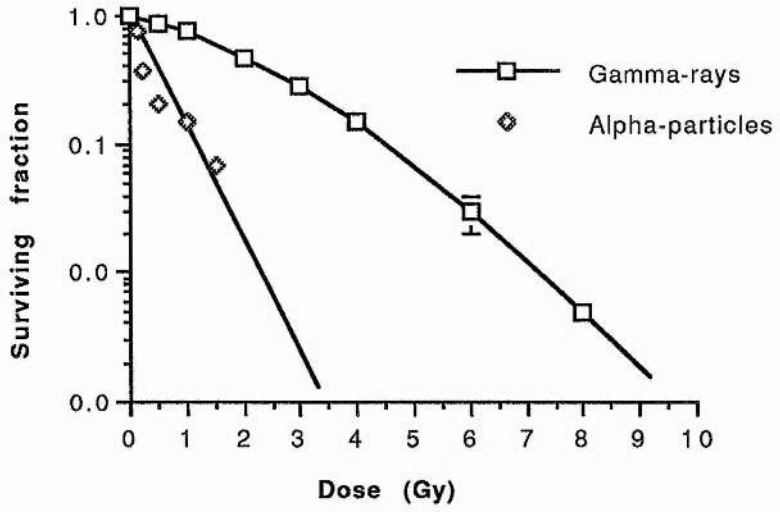


Figure 3.6.5.1. Surviving fraction of HTori3 cells irradiated with graded doses of ^{137}Cs γ-rays and ^{238}Pu α-particles. Results for γ-rays are based on triplicate experiments, while experiments with α-irradiation were unreplicated. Error bars represent standard error of means (S.E.M.).

HT3x2γ was significantly more radiosensitive as compared to the parent HTori3 cell line (F=3.42; df=6; p = 0.008). From the statistical analysis, there was no evidence to suggest that there was a significant difference in the radiosensitivity between any of the other tumour cell lines and the parent HTori3 cells.

3.6.6. Comparison of growth rates in culture flasks

The growth of the HTori3 cell line and a tumour cell line HT3x2γ was compared in culture. Cells (2×10^4) were seeded in 25 cm^2 culture flasks in culture medium supplemented with 7% FCS (see section 2.1.1.) and cell counts were taken daily for a 7 day time period (Figure 3.6.6.1.). There appeared to be a little observable difference in growth rates between

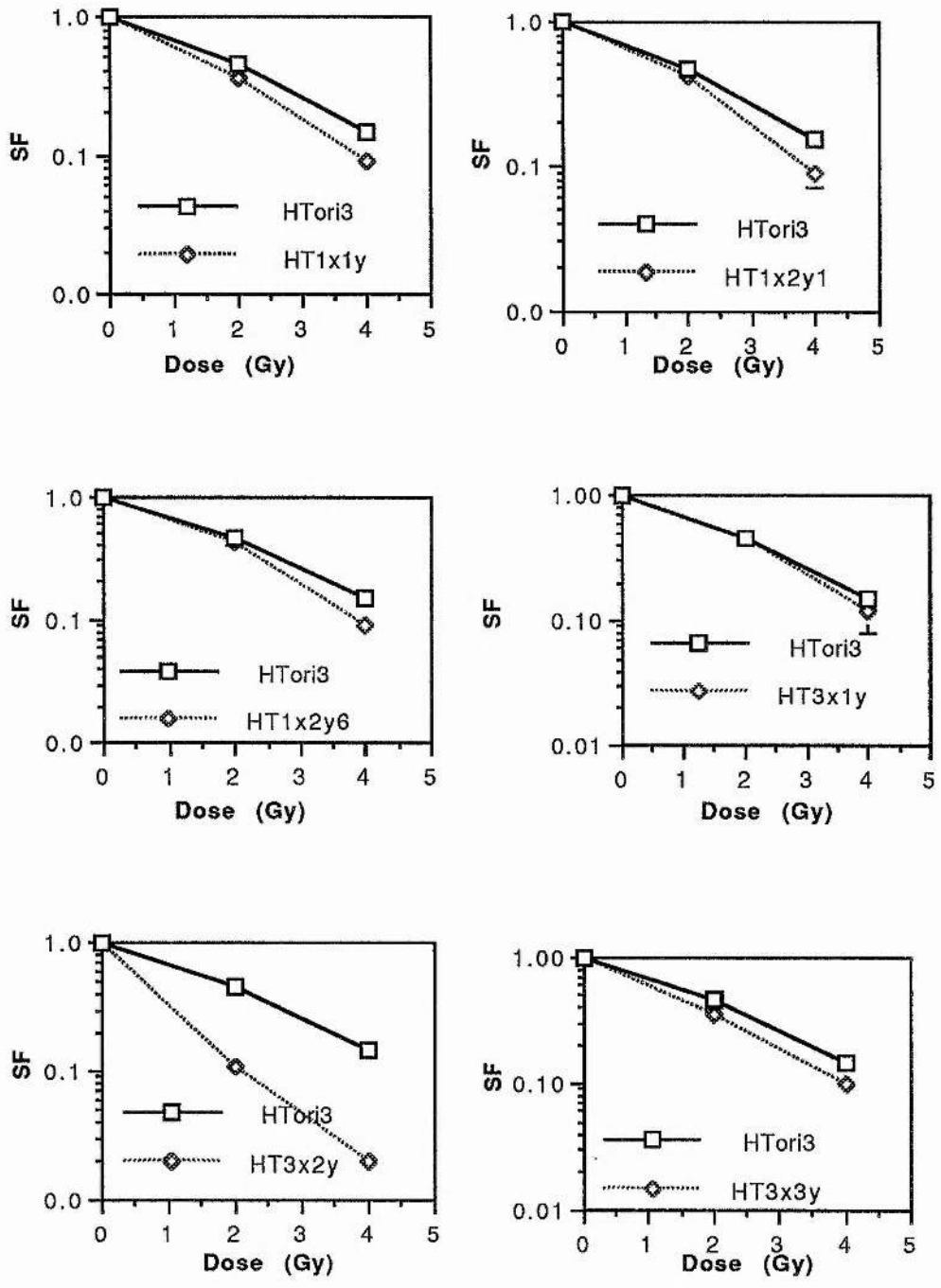


Figure 3.6.5.2. Survival of 6 tumour cell lines compared to HTori3 cells. Data were pooled from 3 experiments. Error bars represent S.E.M.

these two lines between days 1 and 6. However, on day 7 a difference in growth rate was observed.

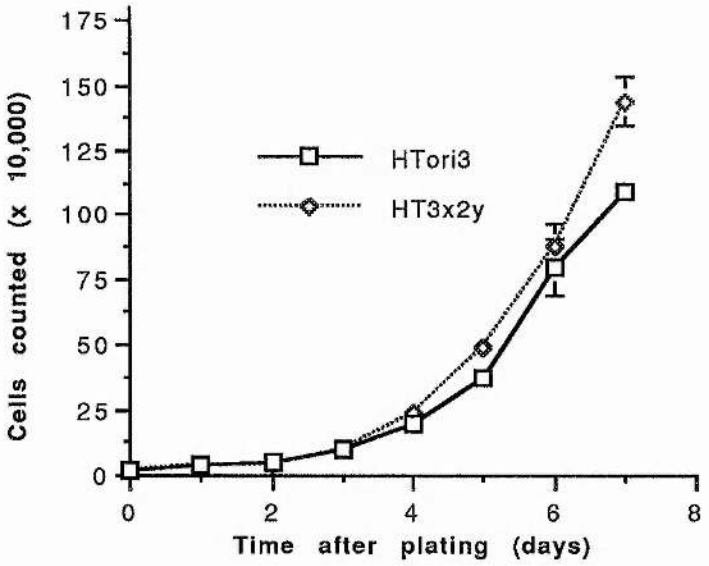


Figure 3.6.6.1. Comparison in growth rates in culture flasks between the parent HTori3 cell line and the tumour cells line HT3x2γ. Error bars represent S.E.M.

3.6.7. Assessment of anchorage dependence of HTori3 cells following γ-irradiation

3.6.7.1. Experimental procedure

HTori3 cells in culture flasks were exposed to 3 doses of 2 Gy γ-irradiation with an interval of 7 days between exposures. Anchorage independent growth were determined following each exposure, and at 2nd and 6th passage after final exposure. Cell suspension (2,000) in 0.3% agar was overlaid in 60 mm culture dishes containing a 0.5% agar underlayer (see section 2.6.4.). Control cultures of unirradiated HTori3

cells were treated identically. The cultures were assayed after a 21 day period for colony formation. Experiments were carried out in duplicate.

3.6.7.2 Results

The results of γ-irradiation on the cloning efficiency of HTori3 cells in soft agar are presented in Figure 3.6.7.1. No statistically significant difference in the cloning efficiency was observed between control and irradiated cultures when tested after 1st, 2nd, 3rd exposure, nor at 2nd passage after final exposure. At 6th passage, however, there was a 2-fold increase in the cloning efficiency of irradiated cells relative to control unirradiated cells.

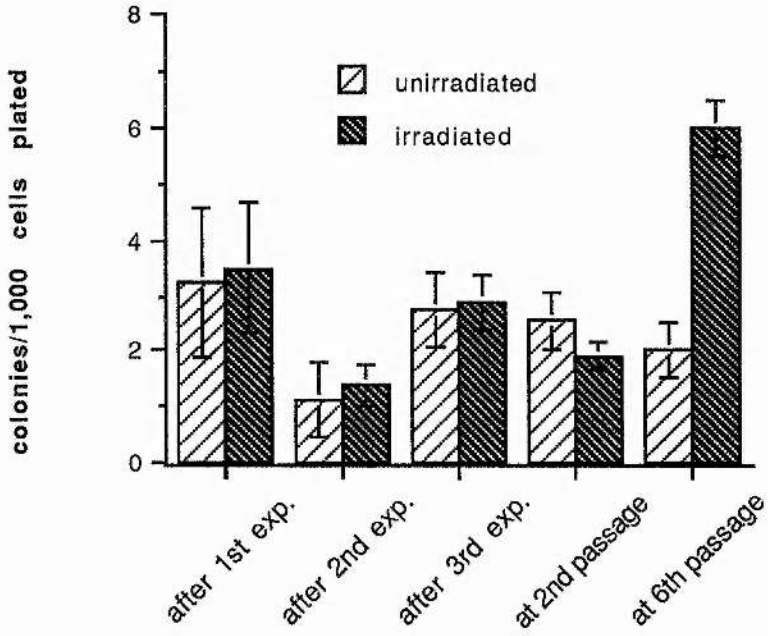


Figure 3.6.7.1. Cloning efficiency of the HTori3 cells in soft agar at different time points following exposure to γ-irradiation.

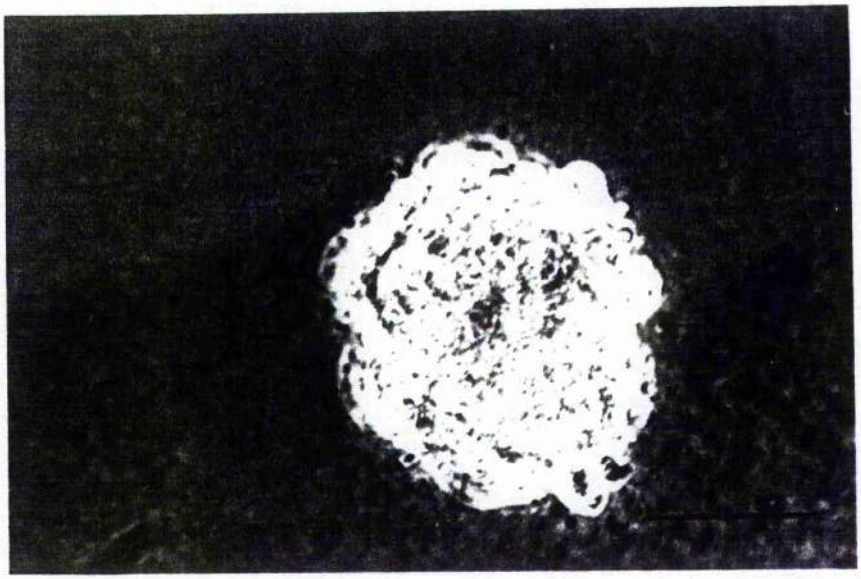
3.6.8. Cloning efficiency of the tumour cell lines in semisolid media

The clonogenicity in semisolid media was assayed. HTori3 cells and a tumour cell line HT3x2γ were seeded in 3% agar and 1.2% methyl cellulose suspension. Cultures were fed weekly and colonies counted following a 28 day incubation (see section 2.6.4.). Both the HTori3 and HT3x2γ cell line showed the ability to form anchorage-independent colonies in both agar and a Methocel suspension. A comparison of the colony-forming efficiency between these two cell lines when grown in soft agar is shown in Table 3.6.8.1. The results suggest that HT3x2γ cells form anchorage-independent colonies at a significantly higher efficiency than HTori3 cells. Figure 3.6.8.1. shows an example of a colony of HTori3 and HT3x1γ cells grown in 3% agar for 21 days. Notice that there is extensive piling up of closely packed cells in the focus, forming a compact colony of more then 0.2 mm diameter.

Table 3.6.8.1. Comparison of plating efficiency in soft agar and on plastic between a tumour cell line HT3x2γ and the parent HTori3 cells. Results were based on 4 experiments. PE = plating efficiency. [a]Anchorage requirement expressed as the percentage of colonies in soft agar to colonies on plastic dishes. [b]Values are mean ± S.E.M.

Cell line	PE on plastic (%)	PE in soft agar (%)	A/P[a]
HTori3	24.36±2.42[b]	0.40±0.09[b]	1.64
HT3x2γ	16.50±2.66	8.75±1.96	53.03

A



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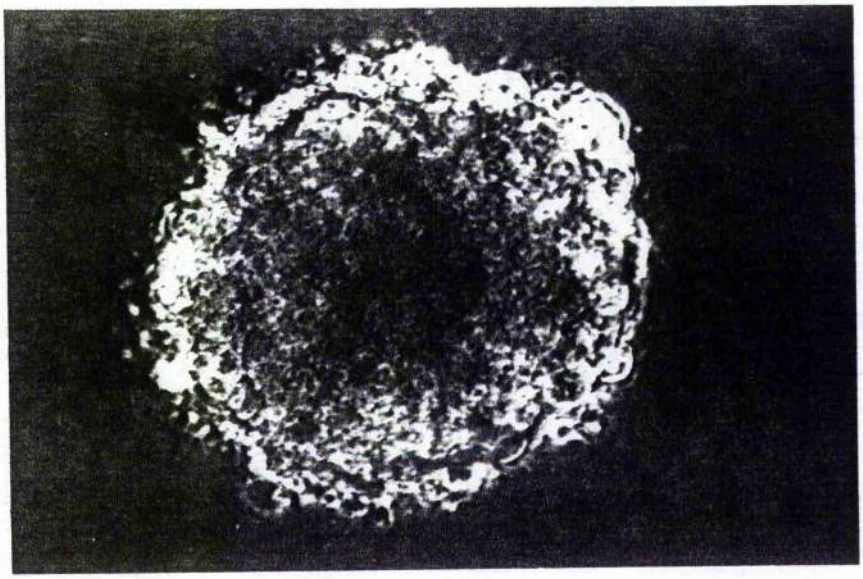

Figure 3.6.8.1. A colony of HTori3 cells (A) and tumour cell line HT3x2γ (B) found in soft agar medium after 21 day incubation. Photograph taken at phase-contrast. Scale bar represents 25 μm.

3.6.9. Analysis of *ras* oncogenes in the radiation-transformed cells

A non-radioactive method was used for the detection of *ras* oncogene mutations in the DNA of cell lines derived from tumours induced by treatment with radiation (see section 2.6.2.). Genomic DNA isolated from the tumour cells was amplified by the PCR reaction, and then digested with specific restriction endonucleases in order to detect RFLP.

3.6.9.1. Detection of H-*ras* mutations

DNA prepared from HTori3 cells, 23 tumour cell lines, as well as the T24 cell line (homozygous for H-*ras* codon 12 mutant valine allele) was amplified using the primers H5' and H3' (see Figure 2.6.2.2.). *Msp I* digestion of PCR amplified DNA detected codon 12 mutations in the positive control T24 cells (Figure 3.6.9.1.). None of the tumour cell lines nor HTori3 had mutations at H-*ras* codon 12 which could be detected by the method described above.

3.6.9.2. Detection of K-*ras* mutations

PCR amplified DNA fragments from HTori3, the tumour cell lines, and a positive control SW480 line (homozygous K-ras codon 12 valine allele mutation) were digested with *BstN*. Examination of the restriction patterns of the DNA indicated codon 12 mutations in the SW480 line but no mutations in HTori3 and the tumour cell lines (Figure 3.6.9.2.).

When PCR amplified K-*ras* DNA was digested with the restriction enzyme *Hph I*, a codon 13 aspartic acid mutation in the positive control HCT116 line was detected. However, negative results were obtained for HTori3 and all tumour cell lines (Figure 2.6.9.3.)

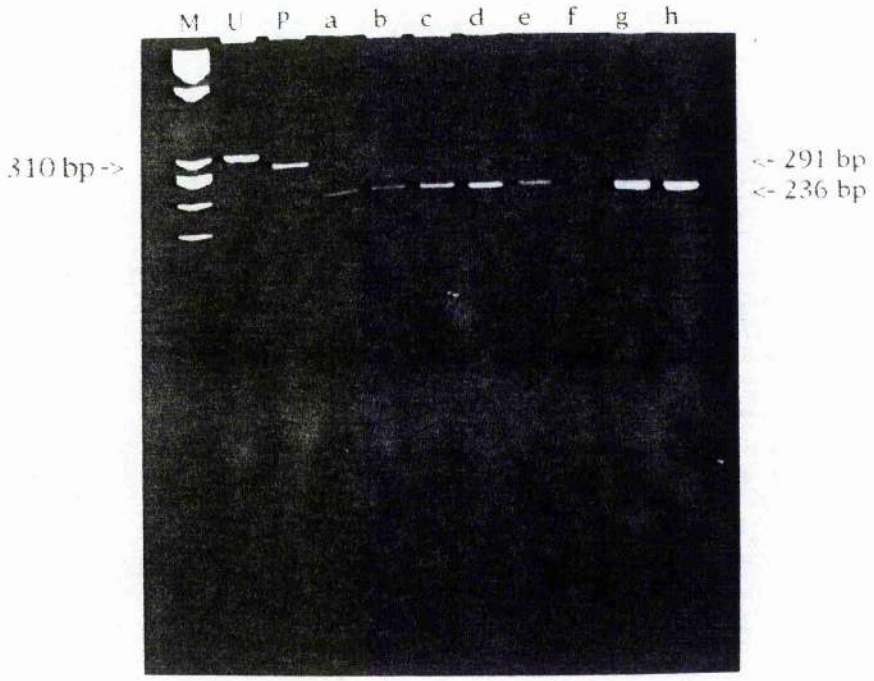

Figure 3.6.9.1.A. Detection of H-ras mutations at codon 12 in the radiation-induced tumour cell lines. Lane M: Hae III digestion of ΦX174 DNA marker; lane U: undigested amplified T24 cell line DNA; lane P: positive control amplified T24 cell line DNA digested by Msp I; lane a: amplified HTori3 DNA digested by Msp I; lanes b-h represent Msp I digests of amplified DNA from radiation-induced tumour cell lines as follows: lane b: HT1x0.125α2; lane c: HT1x0.25α7; lane d: HT1x0.5α8; lane e: HT1x1α4; lane f: HT1x1.5α9; lane g: HT1x1γc; lane h: HT1x2γ7.

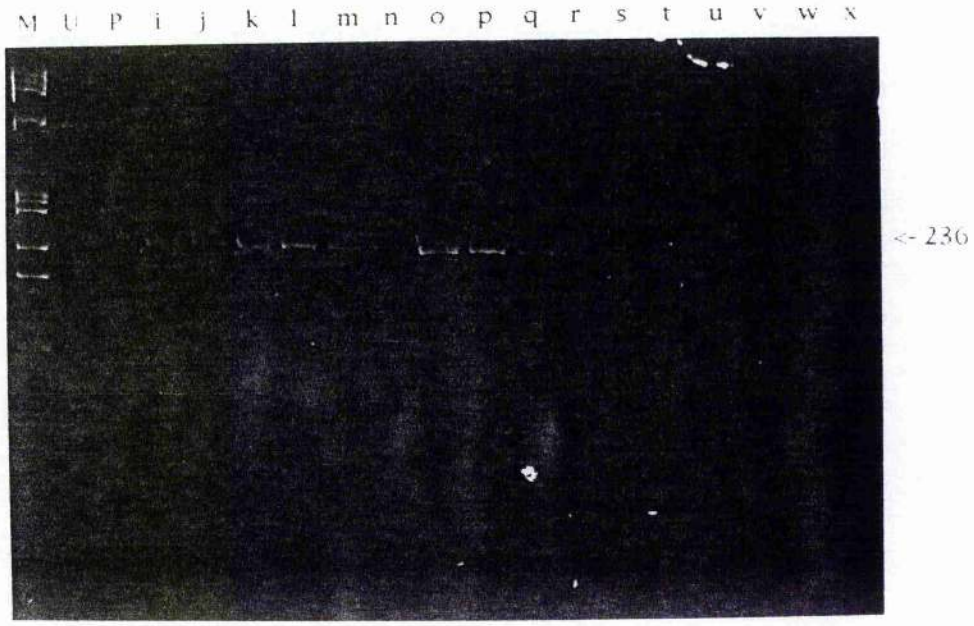

Figure 3.6.9.1.B. Detection of H-ras mutations at codon 12 in the radiation-induced tumour cell lines. Lane M: Hae III digestion of ΦX174 DNA marker; lane U: undigested amplified T24 cell line DNA; lane P: positive control amplified T24 cell line DNA digested by Msp I; lanes i-x represent Msp I digests of amplified DNA from radiation-induced tumour cell lines as follows: lane i: HT1x4γ6; lane j: HT3x1γ; lane k: HT1x0.5α6; lane l: HT1x2γ9; lane m: HT1x0.5α1; lane n: HT1x0.5γT; lane o: HT3x3γ; lane p: HT1x0.25α4; lane q: HT1x0.5α2; lane r: HT1x2γ6; lane s: HT3x2γ; lane t: HT6x1γ; lane u: HT6x3γ; lane v: HT3x2γX; lane w: HT0; lane x: HT1x2γ1.

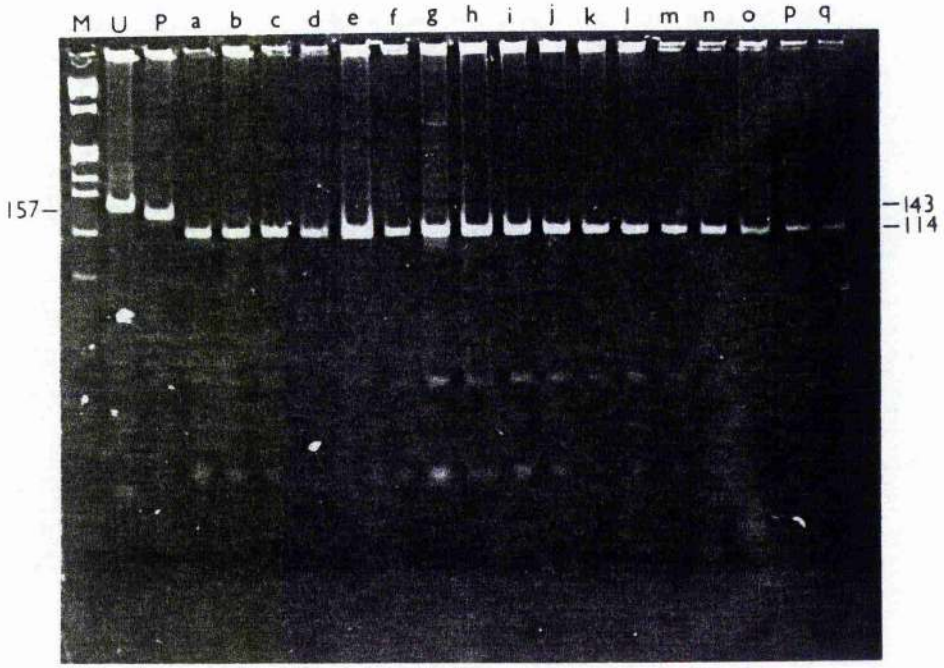

Figure 3.6.9.2.A. Screening of the radiation-induced tumour cell lines for K-*ras* codon 12 mutations. Lane M: Hae III digestion of ΦX174 DNA marker; lane U: undigested PCR amplified SW480 cell line DNA; lane P: positive control amplified SW480 cell line DNA digested by BstN I; lane a: amplified HTori3 DNA digested by BstN I; lanes b-q represent BstN I digests of amplified DNA from radiation-induced tumour cell lines as follows: lane b: HT1x0.125α2; lane c: HT1x0.25α7; lane d: HT1x0.5α8; lane e: HT1x1α4; lane f: HT1x1.5α9; lane g: HT1x1γc; lane h: HT1x2γ7; lane i: HT1x4γ6; lane j: HT3x1γ; lane k: HT1x0.5α6; lane l: HT1x2γ9; lane m: HT1x0.5α1; lane n: HT1x0.5γT; lane o: HT3x3γ; lane p: HT1x0.25α4; lane q: HT1x0.5α2. Numbers on the right and left denote the size of DNA bands in base pairs.

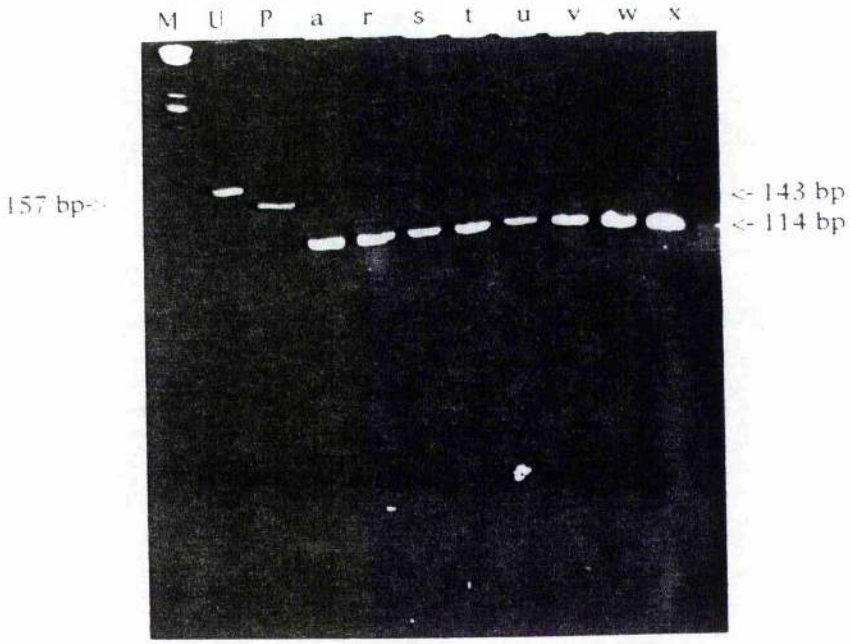

Figure 3.6.9.2.B. Screening of the radiation-induced tumour cell lines for K-*ras* codon 12 mutations. Lane M: Hae III digestion of ΦX174 DNA marker; lane U: undigested PCR amplified SW480 cell line DNA; lane P: positive control amplified SW480 cell line DNA digested by BstN I; lane a: amplified HTori3 DNA digested by BstN I; lanes r-x represent BstN I digests of amplified DNA from radiation-induced tumour cell lines as follows: lane r: HT1x2γ6; lane s: HT3x2γ; lane t: HT6x1γ; lane u: HT6x3γ; lane v: HT3x2γX; lane w: HT0; lane x: HT1x2γ1.

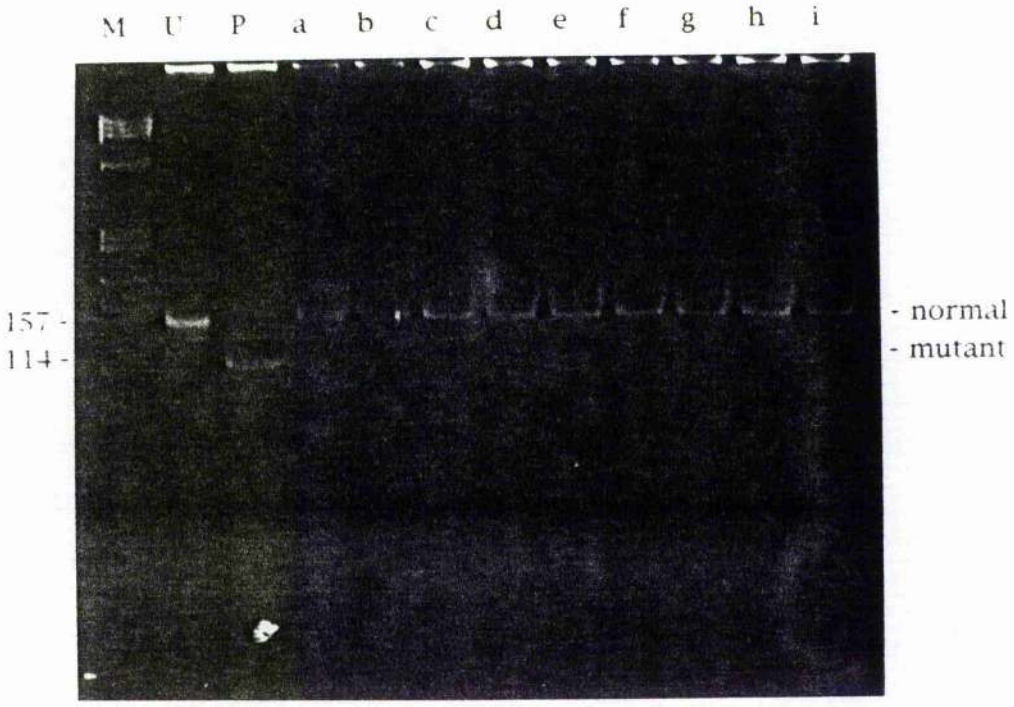

Figure 3.6.9.3.A. Screening of the tumour cell lines for K-*ras* codon13 aspartic acid mutations. Lane M: Hae III digestion of ΦX174 DNA marker; lane U: undigested amplified HTori3 DNA; lane P: amplified HCT116 cell line DNA digested by Hph I (positive control); lane a: amplified HTori3 DNA digested by Hph I; lanes b-i represent Hph I digest of amplified DNA from radiation-induced tumour cell lines as follows: lane b: HT1x0.125α2; lane c: HT1x0.25α7; lane d: HT1x0.5α8; lane e: HT1x1α4; lane f: HT1x1.5α9; lane g: HT1x1γc; lane h: HT1x2γ7; lane i: HT1x4γ6.

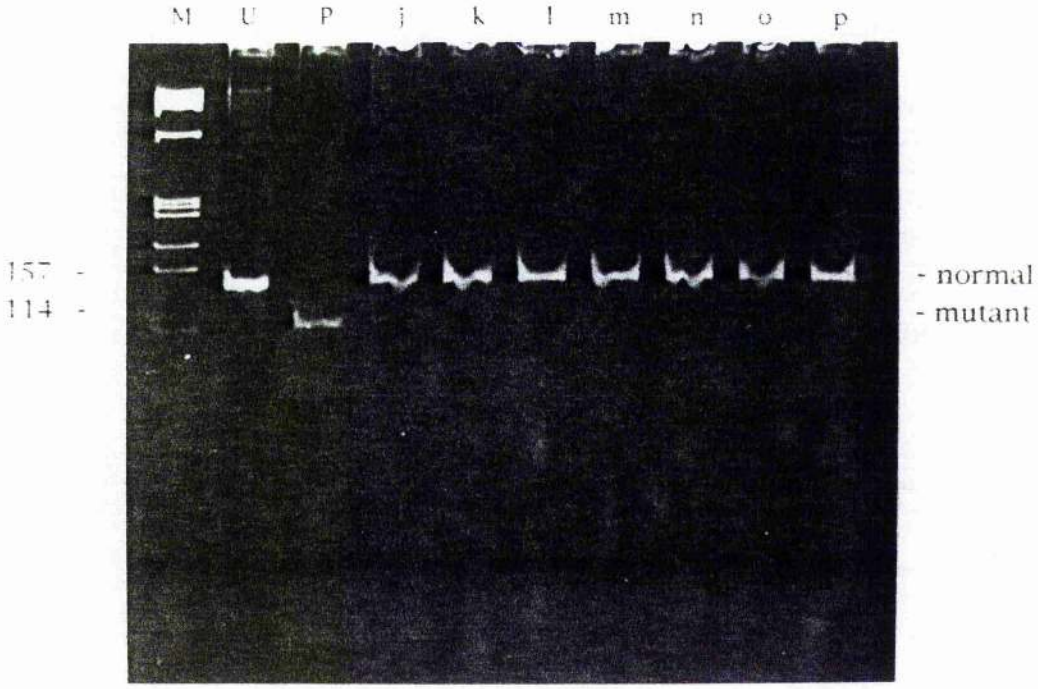

Figure 3.6.9.3.B. Screening of the tumour cell lines for K-*ras* codon13 aspartic acid mutations. Lane M: Hae III digestion of ΦX174 DNA marker; lane U: undigested amplified HTori3 DNA; lane P: positive control amplified HCT116 cell line DNA digested by Hph I; lanes j-p: Hph I digests of amplified DNA from radiation-induced tumur cell lines as follows: lane j: HT3x1γ; lane k: HT1x0.5α6; lane l: HT1x2γ9; lane m: HT1x0.5α1; lane n: HT1x0.5γT; lane o: HT3x3γ; lane p: HT1x0.25α4.

3.6.10. Analysis of p53 protein in the radiation-transformed cells

The normal parental HTori3 cells and the tumour cell lines were examined for the expression of mutant p53 protein. Figure 3.6.10.1. shows a Western blot analysis of the p53 protein in 6 tumour cell lines. Immunoprecipitation of p53 proteins was carried out by two antibodies: DO-1, which recognises both mutant and wild type p53 protein, and PAb 240 which detects only the mutant type.

As a positive control for mutant p53, a human osteosarcoma cell line (HOS) was used, this was beacuse it has a p53 point mutation with an arginine to proline substitution at codon 152. The human bladder carcinoma cell line (T24) was used as a negative control for mutant p53 protein. The lanes probed with Anti-Rb antibody IF8 gave a negative result, demonstrating that there was no non-specific binding by secondary anti-p53 antibody CM-1.

The control and tumour cell lines gave intense positive staining (53 kD band) for p53 with DO-1. In contrast, only very weak bands were detected with PAb 240 antibody in the HTori3 cells and 6 tumour cell lines tested (Figure 3.6.10.1.). However, PCR-SSCP analysis of p53 exons 5-8 was undertaken in collaboration with Dr. S. Gamble and Professor J. Arrand at Brunel University in London. In the tumour cell lines tested p53 mutations at exons 5, 7, and 8 but not exon 6 was detected (Gamble *et al.*, 1995).

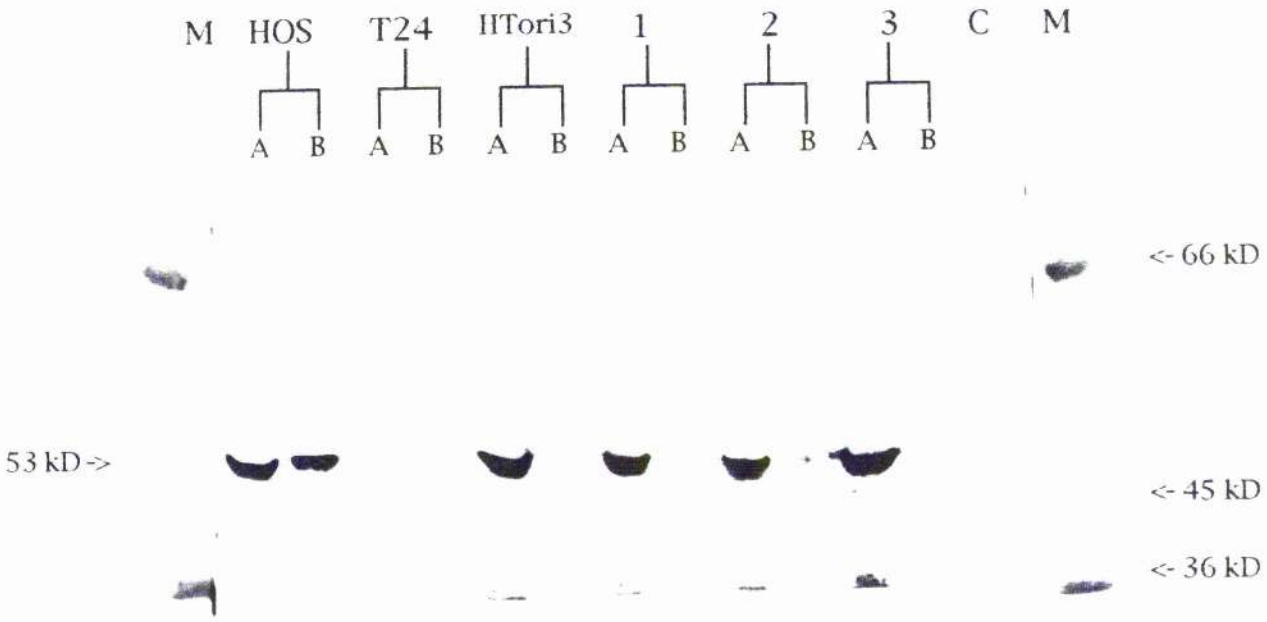

Figure 3.6.10.1.A. Western blot analysis of p53 protein in the radiation-induced tumour cell lines and their nontumorigenic cell precursor HTori3 cells. p53 from cell line extracts were immunoprecipitated with monoclonal antibody DO-1 (A), PAb 240 (B), or irrelevant negative control anti-Rb antibody (C). Immunoblots were probed with rabbit anti-p53 serum CM-1 and visualized using HRP-conjugated swine anti-rabbit immunoglobulins. Lane M: Dalton Mark VII-L protein marker; HOS: human osteosarcoma cell line used as positive control for mutant p53; T24: human bladder carcinoma cell line used as negative control for mutant p53; HTori3: human thyroid epithelial cell line from which the tumour cell lines were derived by irradiation; lane T1: HT1x1α4 tumour cell line; lane T2: HT1x1.5α9 tumour cell line; lane T3: HT1x1γC tumour cell line. Numbers on the right side denote molecular weights of the protein markers.

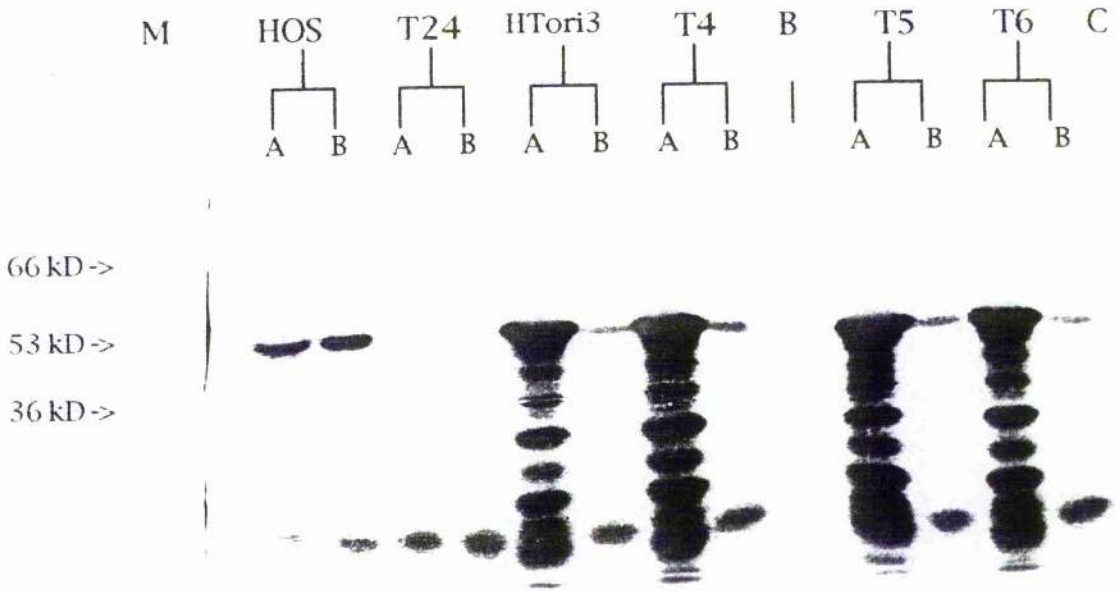

Figure 3.6.10.1.B. Western blot analysis of p53 protein in the radiation-induced tumour cell lines and their nontumorigenic cell precursor HTori3 cells. p53 proteins from cell line extracts were immunoprecipitated with monoclonal antibody DO-1 (A), PAb 240 (B), or anti-Rb antibody (C). Immunoblots were probed with rabbit anti-p53 serum CM-1 and visualized using HRP-conjugated swine anti-rabbit immunoglobulins. Lane M: Dalton Mark VII-L protein marker; HOS: human osteosarcoma cell line used as positive control for mutant p53; T24: human bladder carcinoma cell line used as negative control for mutant p53; HTori3: human thyroid epithelial cell line from which the tumour cell lines were derived by irradiation; lane T4: HT1x0.125α2 tumour cell line; lane B: blank; lane T5: HT0.25α7 tumour cell line.; lane T6: HT1x0.5α8 tumour cell line; lane C: HTori3 cells extract immunoprecipitated with anti-Rb antibody (irrelevant negative control).

Chapter 4

DISCUSSION

Ionizing radiation has been known to produce a number of different DNA lesions in human and animal cells which can lead to cell killing, mutation of a specific gene, and carcinogenesis. Different tissue types have different susceptibility to radiation-induced cancer. Studies have revealed that the thyroid gland has the second highest relative radiosenitivity (after myelopoietic tissue) to the induction of radiogenic cancer (Storer, 1982).

There have been many studies which have suggested an association between radiation and an increased thyroid cancer incidence. A study of children treated with radiation for thymus enlargement (Simpson *et al.*, 1955) and scalp ringworm (Albert & Omran, 1968; Modan *et al.*, 1977; Ron *et al.*, 1989) and cohort studies on siblings in the United States (Hempelmann *et al.*, 1975) showed an increased incidence of thyroid cancer compared to the normal population. Since the Chernobyl accident in 1986, there has been a substantial increase (approximately 80 fold) in the incidence of thyroid carcinoma in children in Southern Belarus and Northern Ukraine (Williams *et al.*, 1993).

The development of a suitable *in vitro* model for radiation-induced neoplastic transformation of human epithelial cells would have a significant importance in both cancer biology and radiation biology. Using the human thyroid follicular epithelial cells as a model for studying neoplastic transformation has two main attractive features. Firstly, most

human malignant tumours are of epithelial origin. Therefore, the human epithelial system may be most relevant in dissecting the process of carcinogenesis in man. Secondly, since the carcinogenic action of ionizing radiation in thyroid cells has been recognized from experimental and epidemiological studies (see above), an *in vitro* model using human thyroid epithelial cells may increase our understanding of the mechanisms underlying radiation-induced carcinogenesis in the thyroid gland.

In the past two decades, rodent cell models have been used extensively in studying neoplastic transformation *in vitro*. These rodent models have been used successfully to quantify the carcinogenic effect of chemical, biological and physical agents.

In contrast, despite many attempts, there has been very little success in developing an *in vitro* model using human epithelial cells. In particular, it has proven to be extremely difficult to achieve neoplastic transformation *in vitro* of human cells by ionizing radiation. There are three reasons for this observations: difficulties in maintaining human cells in culture, the very low transformation frequencies radiation induces in human cells and the problems associated with detecting a transformed phenotype. Morphologic change, the characteristic often associated with neoplasia of rodent cells, does not appear to be easily discernible in human cells after treatment with carcinogens. Transformed rodent cells in culture are identifiable by dense multilayered colonies with random cellular arrangement (Reznikoff *et al.*, 1973a, 1973b). Colony formation in culture dishes, however, is an extremely rare phenomenon amongst *in vitro* transformed human cells. Therefore, human cells differ from rodent cells in that neoplastic transformation *in vitro* is not readily identified by a change in morphology.

It is extremely difficult to immortalize human cells without using DNA viruses such as SV40, adenoviruses and papilloma viruses (Rhim *et al.*, 1990). There have been only a few reports of immortalization of human cells, either spontaneously or as a result of treatment by chemical or physical agents. Interestingly immortalized human cells can be easily converted into neoplastic cells by certain oncogenes, for example *ras* oncogenes (Rhim *et al.*, 1990) but are resistant to other oncogenes, chemical and physical agents (Rhim *et al.*, 1986, Reznikoff *et al.*, 1988). These results indicate that immortalization is a critical initial step for *in vitro* neoplastic transformation of human cells. Therefore in this study, it was necessary to use human thyroid epithelial cells, previously immortalized with SV40. These cells were designated as HTori3 (Lemoine *at al.*, 1989).

The aim of this thesis was to study the neoplastic transformation of human epithelial cells as a result of treatment with ionizing radiation *in vitro*. The study was designed to examine whether *in vitro* exposure to ionizing radiation is able to induce tumorigenic conversion of the non-tumorigenic immortalized human epithelial cells and also how different types of radiation and radiation regimes affect the transformation frequency. In addition, expression time following irradiation was investigated and also the possible existence of markers for identifying the transformed phenotype.

4.1. Transformation studies

The end point used to test for neoplastic transformation was the ability of cells to produce progressively growing tumours in athymic nude mice. The nude mouse model, although not ideal, is the best

available at present and has proven to be a reliable test system (Giovanella *et al.*, 1978).

The results obtained in this study show that HTori3 cells, following exposure to irradiation and subsequent passaging, become tumorigenic, forming progressively growing tumours in athymic nude mice. Statistically significant increases in tumour incidence were obtained with both γ- and α-irradiation and with both single and multiple irradiation regimes as compared with the un-irradiated group. Tumours were also produced when irradiated cells were transplanted immediately following exposure to radiation without subsequent passaging.

It was demonstrated that neoplastic transformation of immortalized human thyroid epithalial cells can be achieved *in vitro* by treatment with ionizing radiation. More importantly, the transformation data further suggests that a single exposure to ionizing radiation is sufficient to cause neoplastic transformation.

Moreover, investigation of expression time following irradiation demonstrated that transplanation of irradiated cells into nude mice at different times after exposure did not result in a significantly different tumour incidence between the groups. These findings contrast with the studies with irradiation using X-rays (Borek & Hall, 1966; Borek & Sachs, 1967; Terzaghi & Little, 1974; 1975; Borek, 1980) and chemicals (Chen & Heidelberger, 1969; Kakunaga, 1975; Milo & DiPaolo, 1978; Milo *et al.*, 1981; Reznikoff *et al.*, 1988; Bookland *et al.*, 1992) which have shown that treated mammalian cells need a certain period of passaging in culture prior to transplantation into nude mice for the fixation of a transformed state. It was not clear if this period of cell proliferation was necessary to permit further expansion of the number of transformed cells, or if further genetic changes may take place durig this period. Chromosome losses and

rearrangements observed in this period suggested that the latter hypothesis may be the case (Reznikoff *et al.*, 1988; Willey *et al.*, 1993).

However, the results of this study suggest that post-irradiation passaging, generally regarded as an indispensible step for fixation and expression of radiation-induced DNA damage, was not a prerequisite for the neoplastic conversion of irradiated cells. It may be speculated that these genetic changes take place upon transplantation into nude mice.

Tumours were generated with all radiation doses and irradiation regimes used except 3 x 4 Gy γ-rays. It is interesting to note that regardless of radiation type (γ- and α-irradiation) or radiation regime (single or multiple doses) there appears to be a trend which can be described by a bell shaped curve. With increasing doses of radiation, tumour incidence increases and reaches maximum, after which the tumour incidence decreases.

However, it is evident that, due to small the sample size for some of the radiation doses, there is not adequate information available to indicate definitively the quantitative relationship between tumour incidence and radiation dose. Therefore, in order to confirm this trend, an additional quantitative relationship between radiation dose and transformation frequency remains to be determined.

In no instance has a linear relationship between radiation dose and neoplastic transformation been demonstrated. A comparison of the dose-response data for transformation obtained with mouse embryo fibroblasts (Terzaghi & Little, 1976) and hamster embryo cells (Borek & Hall, 1973) with those obtained here illustrates one important parallel. The transformation frequency was found to increase up to doses of approximatelly 3 Gy and then reaches a plateau. At doses greater then 3 Gy, data obtained in this study and those of Borek & Hall suggest a decline

in transformation frequency. The tendency for carcinogenic dose-response curves to reach a saturation point and decline at high levels appears to be a consistent phenomenon with many types of neoplasms (Upton, 1961). It is particularly conspicuous in the induction of thyroid tumours induced by ^{131}I in rats *in vivo* (Lindsay *et al.*, 1957; Potter *et al.*, 1960).

A comparison of the transformation frequency with the γ-radiation survival data indicates that at lower doses there is a little decline in survival but an increase in the transformation frequency. A similar relationship between survival and transformation incidence has also been reported previously (Borek & Hall, 1973; Terzaghi & Little, 1976). It has been suggested that there is minimal cell killing at low doses because cells can accumulate a certain amount of damage (sublethal damage) without reproductive cell death. While higher doses of radiation lead to lethal events, lower doses enhance the transformation frequency through enhanced cell survival. This may explain the decline in transformation incidence observed in this study with the doses greater than 3 Gy γ-irradiation.

This explanation may be challenged with a suggestion that essentially every irradiated cell is "initiated" and potentially transformable even by a very low dose of radiation and that a secondary step is a rare critical event (Kennedy *et al.*, 1980; Little, 1981; Kennedy & Little, 1984). This means that the initial number of cells at risk is not a crucial factor in determining the transformation frequency. The results obtained contradict this notion and support the idea that the high cell killing occuring in the high dose range is responsible for the decline in transformation frequency.

Early studies with cultured mouse cell lines suggested that a single exposure of low- or high-LET radiation was sufficient to induce transformants that were tumorigenic *in vivo* (Hall & Hei, 1985; Hill *et al.*, 1985; Yang *et al.*, 1985; Geard *et al.*, 1986; Miller *et al.*, 1989). Experiments with cultured primary hamster embryonic cells yielded similar results (Borek, 1982; Suzuki *et al.*, 1989).

In contrast, studies with human epidermal keratinocytes, mammary epithelial cells (Yang *et al.*, 1991) and bronchial epithelial cells (Willey *et al.*, 1991) suggested that a single exposure of radiation was insufficient to cause full transformation. Cells exposed to single doses of radiation exhibited morphological alterations or anchorage independent growth, but did not form tumours in nude mice. It was therefore suggested that a single exposure to radiation was unlikely to result in human cells becoming tumorigenic, but can however result in the initial step of transformation (Yang, 1991; Rhim *et al.*, 1993).

Results with single doses of α- and γ-radiation obtained in this study indicate that single exposure to radiation can fully transform immortalized human epithelial cells. This is in agreement with reports of the succesful transformation of human bronchial epithelial cells with single exposure of α-particles (Martin *et al.*, 1993; Hei *et al.*, 1994). These findings support the idea that transformation of immortalized human epithelial cells can be achieved by single doses of radiation.

Results indicating that single exposure of radiation can fully transform some human epithelial cells do not necessarily contradict those unsuccessful attempts. These conflicting results may be explained since radiogenic transformation is not only species but also tissue dependent. In addition, the clastogenic effects of radiation may not be limited to the actual time of exposure, but may linger for a period after

irradiation with further accumulation of chromosomal damage. Kadhim *et al.* (1992) demonstrated that haemopoietic stem cells irradiated with α-particles transmit to their daugther cells chromosomal instability that may result in one or more visible cytogenetic aberrations many cell cycles later. The nonclonality and variable number of cells in a colony exhibiting chromosomal aberrations is consistent with them arising *de novo* in cells derived from a clonogenic cell that survived irradiation before the initiation of clonal proliferation. This phenomenon may help to explain succesful neoplastic transformation of HTori3 cells following a single exposure to radiation.

Although, all radiation regimes induced a statistically significant increase in tumour incidence, a small number of tumours was obtained in control unirradiated group in two of the experiments. It should be said, however, that the control group size (92 nude mice inoculated) was considerable larger as compared to the control group size in other studies of radiation-induced transformation of human cells. Only 4 mice (human keratinocytes; Thraves *et al.*, 1990), and 22 mice (human bronchial epithelial cells; Hei *et al.*, 1994) were inoculated with control unirradiated cells. Control group size used in other transformation studies using human cells was not specified (Yang *et al.*, 1991; Martin *et al.*, 1993)

The reasons for the observation of tumours in the control groups can only be speculated. One likely cause of neoplastic conversion in the untreated cells may be linked to p53 protein function. According to the widely accepted hypothesis wild-type p53 has a function as a "molecular guardian" monitoring the integrity of the genome (Lane, 1992). If the DNA is damaged p53 accumulates and switches off replication to allow extra time for its repair. If the repair fails, p53 may trigger cell suicide by apoptosis.

HTori3 cells used in this study were immortalized by SV40 genome. Therefore the biological function of p53, as a result of binding to large T antigen, may have been abolished. These cells may not be able to carry out G1/S arrest and can be regarded as genetically less stable. They may be more susceptible to the accumulation of genetic mutations and chromosomal rearangements at an increased rate, leading to the rare spontaneous selection of the neoplastic phenotype. This hypothesis is supported by studies with p53-deficient mice. They showed that such animals are susceptible to spontaneous tumours (Donehower *et al.*, 1992). This notion would be in accordance with the report of spontaneous neoplastic conversion of SV40 immortalized human epidermal keratinocytes after approximately 46 *in vitro* passages, forming invasive squamous cell carcinomas in athymic nude mice (Brown & Gillimore, 1987).

The development of tumours in the control group in this study, together with previous reports of transformation of human immortalized cells (Tainsky *et al.*, 1984; Brown & Gallimore, 1987), indicates that "silent" genetic changes may have occured spontaneously during the *in vitro* passaging of the immortalised human cells. Therefore HTori3 cells can be regarded as premalignant cells with increased genetic instability which could, after radiation-induced damage, lead to even faster accumulation of the genetic changes which are necessary for a fully transformed phenotype to be achieved.

Although it is not certain how many events are involved, there is a general agreement about the order that these events take place. This study confirms the notion that immortalization is a critical initial and rate-limiting event in the transformation of normal human cells. Thus, to make a practical *in vitro* system with human cells, it is required that the

cells escape from cellular "senescence", since this is the dominant trait in all human cell culture (Hayflik & Moorhead, 1961). Also, normal human cells very rarely undergo spontaneous transformation in culture. This is in direct contrast to rodent cells, where spontaneous transformation occurs frequently (DiPaolo, 1983). The basis for this is not fully understood, several expalanations, such as differences in genetic stability and natural life span, can be speculated (Rhim, 1993).

This study demonstrated that the *in vitro* neoplastic transformation of human epithelial cells can be achieved in two steps: immortalization and neoplastic conversion. These results also demonstrate neoplastic transformation of human epithelial cells *in vitro* can be achieved by incorporating a DNA-viral gene into the cell genome followed by exposure to ionizing radiation. This supports the widely accepted multistep hypothesis of neoplastic transformation of human epithelial cell (Reznikoff *et al.*, 1988; Rhim, 1993). Although, it has been proposed that only one oncogene alone, v-Ha-*ras* (Yoakum *et al.*, 1985), may be sufficient for neoplastic transformation of bronchial epithelial cells *in vitro*, the long time-scale and step-wise methods used to isolate altered cells, suggest that several steps are involved.

4.2. Histological examination of tumours

All tumours grown in nude mice following transplantation of irradiated HTori3 cells were histologicaly examined. Paraffin-embedded sections revealed that the tumours consisted predominantly of areas of poorly differentiated cells with occasional evidence of small follicular areas. Therefore, the tumours were all diagnosed as undifferentiated anaplastic carcinomas.

Wynford-Thomas (1993) has proposed a model which suggest that neoplasia of human thyroid follicular cells progress along two distinct pathways: "follicular" and "papillary" pathway, both arising from the follicular cells, and culminating in the anaplastic carcinoma. This different biological behaviour may be associated with different patterns of oncogene activation (Lemoine *et al.*, 1988; Ishizaka *et al.*, 1989; Grieco *et al.*, 1990; Pierotti *et al.*, 1992; Jhiang *et al.*, 1992; Williams & Williams, 1995).

According to published reports (Kazakov *et al.*, 1992; Beverstock *et al.*, 1992; Furmanchuk *et al.*, 1992; Williams *et al.*, 1993; Palle & Salomaa, 1994) thyroid tumours in children in Belarus and Ukraine observed after the Chernobyl nuclear catastrophe were mostly papillary carcinomas. Although estimated doses received by the children in these two regions (2 - 5 Gy) (Williams *et al.*, 1993) are comparable to doses used in this study (up to 4 Gy) it is striking that thyroid tumours most commonly observed following radiation exposure were papillary carcinomas, which was not observed in the tumours seen in the study described in this thesis. The basis of the difference in pathohistological picture is unknown.

In adults a considerable proportion of thyroid tumours consists of anaplastic carcinomas. On the contrary, anaplastic carcinomas are rarely encountered in children, where papillary carcinoma clearly dominates (Palle & Salomaa, 1994). Whereas anaplastic carcinomas in this study were induced following irradiation of the thyroid follicular cells derived from an adult, papillary carcinomas registered after the Chernoby accident were observed in children. It may be speculated that the observed difference in histological type reflects the difference in age.

4.3. Identification of tumour cell lines

There are a number instances in which workers have clamed the successful transformation of human cells, however They have later proved to be the result of contamination by other cell lines cultured within the laboratory and not as a consequence of exposure to carcinogenic agents (Masters *et al.*, 1988; McCormick *et al.*, 1988; Christensen *et al.*, 1993). These finding emphasize the importance of careful examination of the identity of cells with which one is working.

The cell lines established in this study from primary tumours grown in nude mice upon transplantation of irradiated HTori3 cells were demonstrated to be of human origin by karyotype analysis. Also, the differentiation marker for human epithelial cells, cytokeratin, continued to be expressed in both the parent and radiation-induced tumour cell lines, confirming the epithelial origin of the cell lines. In addition, positive immunohistochemical staining of SV40 large T antigen confirmed that the tumour cell lines share this characteristic with putative parent cells.

However, very strong evidence of cell origin came from the DNA fingerprinting analysis. DNA fingerprint of the parent cell line was compared with those of tumour cell lines derived from it. All eleven resolvable bands observed in the parent line were shared with all tumour cells lines. There was no band sharing with a sample included from other individual for comparison. Jeffreys *et al.* (1985) estimated that a mean probability of band sharing was 0.2. This means that the chances that two cell lines with different origin shared 11 hypervariable DNA fragments

are very low ($0.2^{11} = 2x10^{-8}$). This strongly suggest that all cell lines (except T24 cell line) have the same origin.

However, one tumour cell line showed novel bands. Although a single band could be argued to be due to a genetic change it is unlikely that several band changes can be attributed to random mutational events. It probably indicates that there was a certain number of cells of independent origin. It has been speculated that these novel bands were the result of contamination with mouse fibroblasts during the establishment of the cell line from primary tumours grown in nude mouse. Otherwise these lines shared all other (eleven) resolvable bands with parent line indicating that the majority of the cells were derived from HTori3 cells. Nevertheless, these results clearly provide convincing evidence that all transformed cell lines established are derived from the parent HTori3 cell line.

4.4. Characterization of radiation-transformed tumour cell lines

The morphology of the radiation-transformed cell lines and parent HTori3 cells were compared. The tumour cell lines generally showed some morphological alterations such as heterogeneous appearance and higher saturation densities. Although one solitary focus was observed, no criss-cross or pilling-up growth pattern, which are features of transformed rodent cells (Reznikoff *et al.*, 1973a, 1973b), were observed in the radiation-transformed human thyroid epithelial cell culture. It can therefore be concluded that these cells retained the basic epithelial morphology of parent HTori3 cells.

All of the tumour cell lines established from primary tumours have been re-inoculated into athymic nude mice to determine if the cells retain

their tumorigenic phenotype. Tumours were detected upon inoculation of all tumour cell lines tested. These secondary tumours grew progressively and had significantly shorter latent periods than the primary tumours. This is in accordance with the study with the human uroepithelial cells (Reznikoff *et al.*, 1988; Bookland *et al.*, 1992). The SV-HUC-1 cells, transformed after chemical carcinogen treatment, formed tumours in the nude mice from 8 to 10 weeks up to 36 weeks after transplantation. Cells derived from the primary tumours, when re-inoculated into the nude mice, produced tumours with a shorter latent period than the primary tumours. It was also demonstrated that tumour cell lines produced tumours from lower cell dose challenges giving a clonogenic fraction between 3×10^{-4} and 2×10^{-5} cells (Riches *et al.*, 1994).

Experiments were carried out in order to determine the radiosensitivity of the parent HTori3 cells and of the radiation-transformed tumor cell lines. The shape of survival curve for HTori3 cells obtained with γ-rays is characteristic of that commonly observed for mammalian cells exposed to sparsely ionizing radiation. This curve shows an initial shoulder, implying that γ-radiation are relatively inefficient at killing cells in the low dose region. At higher doses of γ-radiation and all doses of α-particles, the survival curve is a straight line in this semi-logarithmic plot, implying that in this dose range cell survival is an exponential function of dose. This initial shoulder observed with γ-rays suggest the existance of a cell repair capacity and represents a region in which cell killing is minimal.

In order to compare the radiosensitivity of the parent HTori3 cell line with the tumour cell lines derived from it, survival curves were constructed. This was necessary in order to exclude the possibility that the reported transformation was the result of the selection of a pre-existing

transformed subpopulation of the parent cell line. This question arose when a small number of tumours were detected in a control unirradiated group, although at significantly lower frequency than in the irradiated groups. Since statistical analysis suggested that none of the 6 tumour cell lines had a higher radiosensitivity than the parent cells, it seems unlikely that a differential selection process was taking place.

The cloning efficiency of a radiation-induced tumour cell line in semisolid media was significantly higher than that of the parent cell line. However, investigation of the anchorage-independant (AI) growth of HTori3 cells following γ-irradiation did not detect any significant increase in cloning efficiency in soft agar at early stages following irradiation.

AI growth is a phenotype highly characteristic of tumorigenic cells in rodent cell transformation *in vitro*, wherein it is generally considered to be one of the last stages in the progressive acquisition of neoplastic potential (Freedman & Shin, 1974; Stiles *et al.*, 1976; Barrett *et al.*, 1979). Similarly, some human cell systems showed an association between neoplastic transformation and AI growth (Milo & DiPaolo, 1978; Borek, 1980; Sutherland *et al.*, 1980; Silinskas *et al.*, 1981; Tejwani *et al.*, 1981; Maher *et al.*, 1982; Zimmerman & Little, 1983; Rhim *et al.*, 1991; Mihara *et al.*, 1992; Yang & Craise, 1994). However, since most cells cloned in soft agar did not exhibit an indefinite growth potential it was suggested that these cells growing in soft agar were abortively transformed (Namba *et al.*, 1985).

Transformation of other human cells did not show an association with AI phenotype (Freedman & Shin, 1974; Sutherland *et al.*, 1980). These findings, together with the present results, suggest that AI growth does not appear to be an important indicator of neoplastic transformation at early stages.

4.5. Analysis of *ras* oncogenes and p53 protein in the tumour cell lines

The neoplastic transformation of human cells is now thought to be the result of multiple molecular and cellular changes (Weinberg, 1989). Although the mechanisms underlaying this process are poorly understood, it is believed that the inactivation of tumour suppressor genes (Klein, 1987), as well as the activation of cellular proto-oncogenes (Bishop, 1987), are involved. It is interesting that specific genes have been found altered in specific tumours. Moreover, there is evidence suggesting a specificity for activation of particular *ras* gene family members by specific types of carcinogen in some types of tumours. For example, a higher rate of K-*ras* mutation was observed in radiation-associated carcinomas may suggest that radiation may preferentially activate K-*ras* in human cells (Lemoine *et al.*, 1988).

Ionizing radiation has been known to produce a number of different DNA lesions in human and animal cells which can lead to gene mutation, and carcinogenesis. Several reports suggested that radiation may activate cellular proto-oncogenes inducing the neoplastic process. Isolation of a radiation-induced dominant transforming sequence has been reported in both tumorigenic human keratinocytes (Thraves *at al.*, 1990) and in tumorigenic murine fibroblasts (Sawey *et al.*, 1987; Krolewski & Little, 1989). Borek *et al.* (1987) also reported the activation of distinctive transforming genes in radiation-transformed hamster embryo and mouse 10T1/2 cells. However, a specific radiation-induced oncogene has not yet been identified as a causal factor in neoplastic transformation.

There have been several reports on the activation of *ras* oncogenes in radiation-induced neoplasms. Activation of *ras* oncogenes in radiation-induced rodent tumours (Guerrero *et al.*, 1984a, 1984b; Merregaert *et al.*, 1986; Sawey *et al.*, 1987), and lung carcinoma in dogs (Frazier *et al.*, 1986) has also been reported. In addition in rats K-*ras* oncogene activation in radiation-induced thyroid tumours has been reported (Lemoine *et al.*, 1988).

On the other hand, studies on human tumours showed contrasted with these above observations. Studies with radiation-transformed keratinocytes (Thraves *et al.*, 1990; 1993) and radiation-associated lung cuncer (Vahakanagas *et al.*, 1992) suggested no activation of any member of the *ras* family. However, *ras* oncogene activation has been demonstrated in radiation-associated human thyroid tumours (Wright *et al.*, 1991).

In this study PCR amplified DNA from 26 radiation-induced tumour cell lines was tested for the presence of H- and K-*ras* codon 12 mutations, as well as the K-*ras* codon 13 aspartic mutation. No mutation of K- or H-*ras* genes in either of the lines tested has been detected. Although *ras* mutation have been shown to occur in human thyroid tumours (Lemoine *et al.*, 1989), the present finding, together with published data (Thraves *et al.*, 1990; 1993; Vahakanagas *et al.*, 1992), suggest that mutations in H-*ras* or K-*ras* genes may not represent the causal event in radiation-induced neoplastic transformation of human thyroid epithelial cells.

Since mutations in the p53 gene are the most frequently identified genetic change in human cancer (Nigro *et al.*, 1989; Takahashi *et al.*, 1989; Hollstein *et al.*, 1991; Levine *et al.*, 1991; 1994), transformed cell lines were examined for the presence of a mutation in the p53 protein. Parent line,

HTori3, was immortalized with an origin defective SV40 genome, therefore it contained stabilized wild type p53 protein due to the binding to the SV40 large T antigen (Rovinski & Benchimol, 1988).

In this study the immunoprecipitation method was applied to distinguish wild type and mutant p53. To precipitate p53 protein two antibodies were used: DO1 (precipitates both wild and mutant p53) and PAb 240 (recognizes only mutant form of p53 protein). Different point mutations which convert p53 from recessive to a dominant oncogene exert a common conformational effect on the protein which is recognized by PAb240. This conformational change abolishes T antigen binding and promotes self-oligomerization, which suggest a "dominant negative" or "transdominant inhibitory" model for the function of the p53 protein (Gannon *et al.*, 1990; Kuerbitz *et al.*, 1992).

Western blot analysis detected p53 mutation in HOS cell line (positive control) and negative results were obtained with T24 cell line (negative control). Examination of parent HTori3 cells and 6 cell lines derived from radiation-induced tumours revealed intense 53 kDa bands with DO-1 antibody in all cell lines tested. Although weak bands were observed with mutation-specific PAb 240 antibody, these findings can be regarded as negative for p53 mutation.

There are two conflicting reports on association between p53 mutations and human thyroid tumours. Ito *et al.* (1992) reported p53 mutations at exons 5 and 8, and suggested that p53 mutations, in human thyroid gland, play a crucial role in tumour progression. On the other hand, results of analysis of human thyroid tumours obtained by Wright *et al.* (1991) strongly suggested that p53 mutations do not commonly play a role in the pathogenesis of human thyroid carcinoma.

However, since in the present study only a fraction of the tumour lines established were tested, no definate conclusions about the involvement of the p53 gene in radiation-induced neoplastic transformation can be made. Testing of additional cell lines using PCR-SSCP techique are in progress in collaboration with Professor J. Arrand and Dr. S. Gamble (Dept. of Biology and Biochemistry, Brunel University in London). Initial results indicate the existance of p53 mutations in exons 5, 7 and 8 (Gamble *et al.*, 1995). Additional evidence are required in order to get a more comprehensive picture of the p53 status.

Neoplastic transformation of human thyroid epithelial cells described in this study seems to provide a suitable model for investigating molecular and cellular mechanisms underlying radiation-induced transformation of human epithelial cells.

Chapter 5

CONCLUSIONS

◊ The results obtained in the transformation studies demonstrated that human thyroid epithelial cells, which were previously immortalized by SV40 genome, can be further transformed into a tumorigenic phenotype following exposure to ionizing radiation *in vitro*.

◊ Exposure to different radiation types (γ- and α-irradiation) and different irradiation regimes (single and multiple doses) induced neoplastic transformation which was detected in the nude mouse model. Transformation data further suggested that a single exposure to γ- or α-radiation was sufficient to cause neoplastic transformation.

◊ Regardless of the radiation type or the irradiation regime, a bell-shaped dose-response curve was produced. The shape of the survival curve for HTori3 cells obtained with γ-rays is characteristic of that commonly observed for mammalian cells exposed to sparsely ionizing radiation, showing an initial shoulder, implying cell repair. At higher doses of γ-radiation and all doses of α-particles, the survival curve is a straight line, suggesting that in this dose range cell survival is an exponential function of dose.

◊ A comparison of the transformation frequency with the γ-radiation survival data indicates that at lower doses there is little decline in survival, but an increase in the transformation frequency. This suggests that high cell killing occuring in the high dose range may be responsible for the decline in transformation frequency.

◊ Investigation of the expression time following irradiation demonstrated that neoplastic transplantion can be achieved when irradiated HTori3 cells are inoculated into nude mice immediately after exposure. The results of this study thus suggest that post-irradiation passaging, generally regarded as an indispensible step for fixation and expression of radiation-induced DNA damage, was not a prerequisite for neoplastic conversion of irradiated cells.

◊ Cell lines established from tumours grown in nude mice following transplantation of irradiated HTori3 cells were demonstrated to be of human origin by karyotype analysis. The differentiation marker for human epithelial cells, cytokeratin, continued to be expressed in both the parent and radiation-induced tumour cell lines, confirming the epithelial origin of the cell lines. Staining of SV40 large T antigen confirmed that the tumour cell lines share this characteristic with parent HTori3 cells. DNA fingerprinting analysis clearly showed that all transformed cell lines established were derived from the parent HTori3 cell line.

◊ Comparison of cell morphology showed that radiation-transformed tumour cell lines retained the basic epithelial morphology of the parent HTori3 cells, with a heterogeneous appearance and higher saturation density. All of the cell lines established from primary tumours induced by irradiation retained the tumorigenic phenotype in nude mice, producing tumours with a shorter latent period. It was also demonstrated that tumour cell lines produced tumours from lower cell dose challenges.

◊ Survival studies showed that tumour cell lines were not more radioresistant than the parent HTori3 cells. This finding is important in relation to the transformation data, excluding the possibility that observed transformation was the result of selection of pre-existing transformed subpopulations within the parent cell line.

◊ Although the cloning efficiency of radiation-transformed tumour cell lines was higher than that of the parent cells, investigation of anchorage-independent growth of HTori3 cells did not detect a significant increase in cloning efficiency in soft agar at early stages following γ-irradiation. This data indicates that anchorage-independent growth is not an important indicator of neoplastic transformation in human epithelial cells at early stages.

◊ RFLP analysis of PCR amplified DNA from radiation-induced tumour cell lines detected no mutations at H- and K-*ras* codon 12, nor at the K-*ras* codon 13, suggesting that mutations in H-*ras* or K-*ras* genes may not be the causal event of radiation-induced neoplastic transformation in human thyroid epithelial cells.

◊ No mutations in the p53 protein was observed in tumour lines tested. These results are comparable with the published findings which suggest that p53 mutations do not commonly play a role in the pathogenesis of human thyroid carcinogenesis.

◊ This study demonstrated that the *in vitro* neoplastic transformation of human epithelial cells can be achieved in two steps: 1) immortalization, and 2) neoplastic conversion. This supports the widely accepted multistep hypothesis of neoplastic transformation of human cells.

◊ Neoplastic transformation of human thyroid epithelial cells described in this study seems to provide a suitable model for investigating molecular and cellular mechanisms underlying radiation-induced transformation of human epithelial cells.

REFERENCES

Adams R.I.P., Knowler J.T., Leader D.P. (1986) *The Biochemistry of Nucleic Acids*. Chapman and Hall Publishers, London

Albert R.E., Omran A. (1968) Follow-up study of patients treated by X-ray epilation for tinea capitis. I. Population characteristics, post-treatment illnesses and mortality expirience. *Arch. Environ. Health*, **17**, 899-918

Alberts B., Bray D., Lewis J., Raff M., Watson J.D. (1991) *The Molecular Biology of the Cell*. Garland Publishing, New York

Amstad P., Reddel R.R., Pfeifer A., Malan-Shibley L., Mark G.E., Harris C.C. (1988) Neoplastic transformation of a human bronchial epithelial cell line by a recombinant retrovirus encoding viral Harvey *ras*. *Mol. Carcinogenesis*, **1**, 151-160

Archer M.C. (1992) Chemical Carcinogenesis. In: *The Basic Science of Oncology*. Tannock I.F., Hill R.P (eds). McGraw-Hill, Inc., New York, pp 102-116

Balmain A., Pragnell I.B. (1983) Mouse skin carcinomas induced *in vivo* by chemical carcinogens have a transforming Harvey-*ras* oncogene. *Nature*, **303**, 72-74

Barbacid M. (1986) Mutagens, oncogenes and cancer. *Trends Genet.*, **2**, 188-192

Barrett J.C. (1991) Comparison of human versus rodent cell transformation: importance of cell aging. In: *Neoplastic Transformation in Human Cell Culture: Mechanisms of Carcinogenesis*. Rhim J.S., Dritschilo A. (eds) Humana Press, Totowa, NJ, pp 3-13

Barrett J.C., Crawford B.D., Mixter L.O., Schechtman L.M., Ts'o, P.O.P., Pollack R. (1979) Correlation of *in vitro* growth properties and tumorigenicity of Syrian hamster cell lines. *Cancer Res.*, **39**, 1504-1510

(BEIR) National Research Council (U.S.) Advisory Committee on the Biological Effects of Ionizing Radiation (1972). *The Effects on Populations of Exposure to Low Levels of Ionizing Radiation.* Natl. Acad. Sci., Natl. Res. Council, Washington, DC, 1-217

Berthon P., Goubin G., Dutrillaux B., Degeorges A., Faille A., Gespach C., Calvo F. (1992) Single-step transformation of human breast epithelial cells by SV40 large T oncogene. *Int. J. Cancer*, **52**, 92-97

Bettega D., Calzolari P., Chiorda G.N., Tallone-Lombardi L. (1992) Transformation of C3H 10T1/2 cells with 4.3 MeV α-particles at low doses: effects of single and fractionated doses. *Radiat. Res.*, **131**, 66-71

Beverstock K., Egloff B., Pinchera A., Ruchti C., Williams D. (1992) Thyroid cancer after Chernobyl. *Nature*, **359**, 21-22

Bishop J.M. (1983) Cellular oncogenes and retroviruses. *Ann. Rev. Biochem.*, **52**, 301-354

Bishop J.M. (1987) The molecular genetics of cancer. *Science*, **235**, 305-311

Blair D.G., Cooper C.S., Oskarsson M.K., Eader L.A., Wande Woude G.F. (1982) New method for detecting cellular transforming genes. *Science*, **218**, 1122-1125

Bol S., Van den Engh G., Visser J. (1977) A technique for staining haemopoietic colonies in agar cultures. *Exp. Hematol.*, **5**, 551-553

Bond V.P., Cronkite E.P., Lippincott S.W., Shellabarger C.J. (1960) Studies on radiation-induced mammary gland neoplasia in the rat: Relation of the neoplastic response to dose of total-body radiation. *Radiat. Res.*, **12**, 276-285

Bonicel A., Mariaggi N., Hughes E., Teoule R. (1980) *In vitro* γ-irradiation of DNA: Identification of radio induced chemical modification of the adenine moiety. *Radiat. Res.*, **83**, 19-26

Bookland E.A., Swaminathan S., Oyasu R., Gilchrist K., Lindstrom M., Reznikoff C.A. (1992) Tumorigenic transformation and neoplastic progression of human uroepithelial cells after exposure *in vitro* to 4-aminobiphenyl or its metabolites. *Cancer Res.*, **52**, 1606-1614

Borek C. (1980) X-ray-induced *in vitro* neoplastic transformation of human diploid cells. *Nature*, **283**, 776-778

Borek C. (1982) Radiation oncogenesis in cell culture. *Adv. Cancer Res.*, **37**, 159-232

Borek C., Hall E.J. (1973) Transformation of mammalian cells *in vitro* by low doses of X-rays. *Nature*, **243**, 450-453

Borek C., Hall E.J. (1974) Effect of split doses of X rays on neoplastic transformation of single cells. *Nature*, **252**, 499-501

Borek C., Ong A., Mason H. (1987) Distinctive transforming genes in X-ray-transformed mammalian cells. *Proc. Natl. Acad. Sci. USA*, **84**, 794-798

Borek C., Sachs L. (1966) *In vitro* cell transformation by X-irradiation. *Nature*, **210**, 276-278

Borek C., Sachs L. (1967) Cell sucseptibility to transformation by X-irradiation and fixation of the transformed state. *Proc. Natl. Acad. Sci. USA*, **57**, 1522-1527

Boukamp P., Stanbridge E.J., Foo D.Y., Cerutti P.A., Fusenig N.E. (1990) c-Ha-*ras* oncogene expression in immortalized human keratinocytes (HaCaT) alters drowth potential *in vivo* but lacks correlation with malignancy. *Cancer Res.*, **50**, 2840-2847

Breimer L.H. (1984) Enzymatic excision from g-irradiated polydeoxyribonucleotides of adenine residues whose imidazole rings have been ruptured. *Nucleic Acids Res.*, **12**, 6359-6367

Breimer L.H. (1988) Ionizing radiation-induced mutagenesis. *Br. J. Cancer*, **57**, 6-18

Breimer L.H., Lindahl T. (1985a) Thymidine lesions produced by ionizing radiation in double-stranded DNA. *Biochemistry*, **24**, 4018-4022

Breimer L.H., Lindahl T. (1985b) Enzymatic excision of DNA bases damaged by exposure to ionizing radiation or oxidizing agents. *Mutat. Res.*, **150**, 85-89

Brown K., Quintanilla M., Ramsden M., Kerr I.B., Young S., Balmain A. (1986) V-*ras* genes for Harvey and BALB murine sarcoma viruses can act as initiators of two-stage mouse skin carcinogenesis. *Cell*, **46**, 447-456

Brown K.W., Gallimore P.H. (1987) Malignant progression of an SV40-transformed human epidermal keratinocyte cell line. *Br. J. Cancer*, **56**, 545-554

Bryant P.E. (1984) Enzymatic restriction of mammalian cell DNA using *Pvu II* and *Bam H1*: Evidence for double strand break origin of chromosome aberrations. *Int. J. Radiat. Biol.*, **46**, 57-65

Bryant P.E. (1988) Use of restriction enzymes to study the relationship between DNA double strand breaks, chromosome aberrations and other end points in mammalian cells. *Int. J. Radiat. Biol.*, **54**, 869-890

Bryant P.E. (1989) Restriction enzymes and radiation induced double strand DNA breaks and chromosome aberration. Chromosome aberrations, basic and applied aspects. Obe G., Natarajan T.A. (eds)

Bryant P.E., Riches A.C. (1989) Oncogenic transformation of murine C3H 10T1/2 cells resulting from DNA double-strand breaks induced by a restriction endonuclease. *Br. J. Cancer*, **60**, 852-854

Cadet J., Berger M. (1985) Radiation-induced decomposition of the purine bases within DNA and related model compounds. *Int. J. Radiat. Biol.*, **47**, 127-143

Campo M.S., Cameron I.R., Rogers M.E. (1978) Tandem integration of complete and defective SV40 genome in mouse-human somatic cell hybrids. *Cell*, **15**, 1411-1426

Cao J., Wells R.L., Elkind M.M. (1992) Enhanced sensitivity to neoplastic transformation by Cs-137 γ-rays of cells in the G2-/M-phase age interval. *Int. J. Radiat. Biol.*, **62**, 191-199

Capon D., Chen E., Levinson A., Seeburg P., Goeddel D. (1983a) Complete nuceleotide-sequences of the *T24* human bladder-carcinoma oncogene and its normal homolog. *Nature*, **302**, 33-37

Capon D., Seeburg P., McGrath J., Hayflick J., Edman U., Levinson A., Goeddel D. (1983b) Activation of Ki-*ras* 2 gene in human-colon and lung carcinoma by 2 different point mutations. *Nature*, **304**, 507-513

Carter R., Wetton J., Parkin D. (1989) Improved genetic fingerprinting using RNA probes. *Nucl. Acids Res.*, **17**, 5867

Cerutti P.A. (1976) DNA base damage induced by ionizing radiation. In: *Photochemistry and Photobiology of Nucleic Acids*. Wang S.Y. (ed) Academic Press, New York, pp 375-383

Chang S.E. (1986) *In vitro* transformation of human epithelial cells. *Biochemica et Biophysica Acta*, **823**, 161-194

Chehab F.F., Doherty M., Cai S., Kan Y.W., Cooper S., Rubin E.M. (1987) Detection of sicle-cell anemia and thalassemias. *Nature*, **329**, 293-294

Chen T.T., Heidelberger C. (1969) Cultivation *in vitro* of cells derived from adult C3H mouse ventral prostate. *J. Nat. Cancer Inst.*, **42**, 903-914

Chiu S., Oleinick N.L.L., Friedman R., Stambrook P.J. (1982) Hypersensitivity of DNA in transcriptionally active chromatin to ionizing radiation. *Biochemica et Biophysica Acta*, **699**, 15-21

Christensen B., Hansen C., Debiec-Rychter M., Kieler J., Ottensen S., Schmidt J. (1993) Identity of tumorigenic human urothelial cell lines and "spontaneously" transformed sublines. *Br. J. Cancer*, **68**, 879-884

Christian B.C., Kao C., Wu W., Meisner L.F., Reznikoff C.A. (1988) Transformation of SV40-immortalized human urinary tract epithelial cells by transfection with *ras* oncogene. *Proc. Am. Assoc. Cancer Res.*, **29**, 459

Clark R., Stampfer M.R., Milley R., O'Rourke E., Walen K.H., Kriegler M., Kopplin J., McCormick F. (1988) Transformation of human mammary epithelial cells by oncogenic retroviruses. *Cancer Res.*, **48**, 4689-4694

Cohen J.B., Levinson A.D. (1988) A point mutation in the last intron responsible for increased expression and transforming activity of the c-Ha-*ras* oncogene. *Nature*, **334**, 119-124

Collins S., Groudine M. (1982) Amplification of endogenous *myc*-related DNA sequences in human myeloid leukaemia cell line. *Nature*, **298**, 679-681

Cooper G.M., Okenquist S., Silverman L. (1980) Transforming activity of DNA of chemically transformed and normal cells. *Nature*, **284**, 418-421

Coquerelle T., Bopp A., Kessler B., Hagen U. (1976) Strand breaks and the 5'end groups in DNA in irradiated thymocytes. *Int. Natl. J. Radiat. Biol.*, **24**, 397-404

Cox R., Masson W.K. (1978) Do radiation-induced thioguanine-resistant mutants of cultured mammalian cells arise by HGPRT gene mutation or X-chromosome rearrangement? *Nature*, **276**, 629-630

Croce C.M. (1977) Assignment of the integration site for simian virus 40 to chromosome 17 in GM 54VA, a human cell line transformed by simian virus 40. *Proc. Natl. Acad. Sci. USA*, **74**, 315-318

Croce C.M., Girardi A.J., Koprowski H. (1973) Assignment of the T-antigen of simian virus 40 to human chromosome c-7. *Proc. Natl. Acad. Sci. USA*, **70**, 3617-3620

DeCaprio J.A., Ludlow J.W., Figge J., Shew J-Y., Huang C-M., Lee W-H., Marsilio E., Paucha E., Livingston D.M. (1988) SV40 large tumor antigen forms a specific complex with the product of the retinoblastoma susceptibility gene. *Cell*, **54**, 275-283

Deeble D.J., von Sonntag C. (1984) Radiolysis of poly(U) in aqueous solution. The role of primary sugar and base radicals in the release of undamaged uracil. *Int. J. Radiat. Biol.*, **49**, 927-936

de The H., Chomienne C., Lanotte M., Degos L., Dejean A. (1990) The t(15;17) translocation of acute promyelocytic leukaemia fuses the retinoic acid receptor alpha gene to a noveltranscribed locus. *Nature*, **347**, 558-561

DiPaolo J.A. (1983) Relative difficulties in transforming human and animal cells *in vitro*. *J. Natl. Cancer Inst.*, **70**, 3-8

DiPaolo J.A., Woodworth C.D., Popescu N.C., Notario V., Doniger J. (1989) Induction of human cervical squamous cell carcinoma by sequential transfection with human papillomavirus 16 DNA and viral Harvey *ras*. *Oncogene*, **4**, 395-399

Donehower L.A., Harvey M., Slagle B.L., McArthur M.J., Montgomery C.A., Butel J.S., Bradley A. (1992) Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumors. *Nature*, **356**, 215-221

Durst M., Gallahan D., Jay G., Rhim J.S. (1989) Glucocorticoid-enhanced neoplastic transformation of human keratinocytes by human papillomavirus type 16 and activated *ras* oncogene. *Virology*, **73**, 767

Dutt K., Scott M., Del Monte M., Agarwal N., Sternberg P., Srivastava S.K., Srinivasan A. (1990) Establishment of human retinal pigment epithelial cell lines by oncogene. *Oncogene*, **5**, 195-200

Elkind M.M. (1984) Repair processses in radiation biology. *Radiat. Res.*, **100**, 425-449

Endo A., Kano Y., Mihara K., Ohira K., Namba M. (1993) Alteration in the retinoblastoma gene associated with immortalization of human fibroblasts treated with ^{60}Co γ-rays. *J. Cancer Res. Clin. Oncol.*, **119**, 522-526

Farber E. (1984) The multistep nature of cancer development. *Cancer Res.*, **44**, 4217-4223

Farber E., Cameron R. (1980) the sequential analysis of cancer development. *Adv. Cancer Res.*, **35**, 125-226

Farmer G., Bargonetti J., Zhu H., Friedman P., Prywes R., Prives C. (1992) Wild type p53 activates transcription *in vitro*. *Nature*, **358**, 83-86

Fasano O., Birnbaum D., Edlund L., Fogh J., Wigler M. (1984) New human transforming genes detected by a tumorigenicity assay. *Mol. Cell. Biol.*, **4**, 1695-1705

Fearon E.R., Vogelstein B. (1990) A genetic model for colorectal tumorigenesis. *Cell*, **61**, 759-767

Fields S., Jang S.K. (1990) Presence of a potent transcription activating sequence in the p53 protein. *Science*, **249**, 1046-1049

Frankenberg D. (1969) A ferrous sulphate dosemeter independent of photo energy in the range 25 keV to 50 MeV. *Phys. Med. Biol.*, **14**, 597-605

Frankenberg-Schwager M. (1989) Review of repair kinetics for DNA damage induced in eukaryotic cells *in vitro* by ionizing radiation. *Radiotherapy and Oncology*, **14**, 307-320

Frazier M.E., Lindberg R.A., Mueller D.M., Gee A., Seed T.M. (1986) Oncogene involvment in plutonium induced carcinogenesis. Workshop on cell transformation in radiobiology. *Int. J. Radiat. Biol.*, **49**, 542-543

Freedman V.H., Shin S. (1974) Cellular tumorigenicity in nude mice: correlation with cell growth in semi-solid medium. *Cell*, **3**, 355-359

Freyer G.A., Sidik K., Feinstein J., Martin S.G. (1993) The molecular basis of transformation in human cells exposed to alpha particles. In: *Proceedings of the International Symposium on Molecular Mechanisma of Radiation and Chemical Carcinogen-Induced Cell Transformation*. p 4 (Abstract)

Friend S.H., Bernards R., Rogelj S., Weinberg R.A., Rapaport J.M., Albert D.M., Dryja T.P. (1986) A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma. *Nature*, **323**, 643-646

Fung Y., Murphree A., T'Ang A., Qian J., Hindrichs, Benedict W. (1987) Structural evidence of the human retinoblastoma gene. *Science*, **236**, 1657-1661

Furmanchuk A.W., Averkin J.I., Egloff B., Ruchti C., Abelin T., Schappi W., Korotkewich E.A. (1992) Pathomorphological findings in thyroid cancers of children from the Republic of Belarus: a study of 86 cases occuring between 1986 ("post-Chernobyl") and 1991. *Hystopathology*, **21**, 401-408

Gamble S., Riches A., Herceg Z., Briscoe T., Bryant P., Colucci S., Arrand J.E. (1995) Incidence of p53 mutation in low dose and low dose-rate irradiated human epithelial cell lines. In: *Proceedings of Annual Meeting of Association for Radiation Research.* (Abstract)

Gannon J.V., Greaves R., Iggo R., Lane D.P. (1990) Activating mutations in p53 produce a common conformational effect. A monoclonal antibody specific for the mutant form. *EMBO J.*, **9**, 1595-1602

Gannt R., Sanford K.K., Parshad R., Price F.M., Peterson W.D., Rhim J.S. (1987) Enhanced G2 chromatid radiosensitivity, an early stage in the neoplastic transformation of human epidermal keratinocytes in culture. *Cancer Res.*, **47**, 1390-1397

Geard C.R., Georgeson M., Tricisano M. (1986) The mouse 3T3 cell transformation system: mechanism and split dose studies with high and low LET radiation. *Int. J. Radiat. Biol.*, **49**, 518-519

Giovanella B.C., Stehlin J.S., Williams L.J., Lee S.S., Shepard R.C. (1978) Heterotransplantation of human cancer into nude mice. *Cancer*, **42**, 2269-2281

Girardi A.J., Jensen F.C., Koprowski H. (1965) SV40-induced transformation of human diploid cells: crisis and recovery. *J. Cell. Comp. Physiol.*, **65**, 69-84

Gluzman Y., Sambrook J., Frisque R.J. (1980) Expression of early genes of origin-defective mutants of simian virus 40. *Proc. Natl. Acad. Sci. USA*, **77**, 3898-3902

Goldfarb M., Shimizu K., Perucho M., Wigler M. (1982) Isolation and preliminary characterization of a human transforming gene from T24 bladder carcinoma cells. *Nature*, **296**, 404-409

Goodhead D.T., Bance D.A., Stretch A., Wilkinson R.E. (1991) A versatile plutonium-238 irradiator for biological studies with α-particles. *Int. J. Radiat. Biol.*, **59**, 195-210

Graham F.L., van der Eb (1973) A new technique of the assay of infectivity of human adenovirus 5 DNA. *Virology*, **52**, 456-467

Gray L.H. (1965) *Cellular Radiation Biology*. William and Wilkins, Baltimore

Grieco M., Santoro M., Berlingieri M.T., Melillo R.M., Donghi R., Bongarzone I., Pierotti M.A., Della Porta G., Fusco A., Vecchio G. (1990) PTC is a novel rearranged form of the *ret* proto-oncogene and is frequently detected *in vivo* in human thyroid papillary carcinomas. *Cell*, **60**, 557-563

Guerrero I., Villasante A., Corces V., Pellicer A. (1984a) Activation of a c-K-*ras* oncogene by somatic mutation in mouse lymphomas induced by gamma radiation. *Science*, **225**, 1159-1162

Guerrero I., Villasante A., D'Eustachio P., Pellicer A. (1984b) Isolation, characterization, and chromosome assignment of mouse N-*ras* gene from carcinogen-induced thymic lymphoma. *Science*, **225**, 1041-1043

Hall E. (1978) *Radiobiology for Radiologist*. Harper and Row Publishers, London

Hall E. (1988) *Radiobiology for the Radiologist*. Lippincott, Philadelphia

Hall E.J., Hei T.K. (1985) Oncogenic transformation with radiation and chemicals. *Int. J. Radiat. Biol.*, **48**, 1-18

Han A., Hill C.K., Elkind M.M. (1980a) Neoplastic transformation of 10T1/2 cells by ^{60}Co γ-rays: evidence of repair of damage at reduced dose rate. *Int. J. Radiat. Biol.*, **37**, 585-589

Han A., Hill C.K., Elkind M.M. (1980b) Repair of cell killing and neoplastic transformation at reduced dose rates of ^{60}Co γ-rays. *Cancer Res.*, **40**, 3328-3332

Han A., Elkind M.M. (1979) Transformation of mouse C3H/10T1/2 cells by single and fractionated doses of X-rays and fission neutrons. *Cancer Res.*, **39**, 123-130

Hanawalt P.C., Cooper P.K., Ganesan A.K., Smith C.A. (1979) DNA repair in bacteria and mammalian cells. *Ann. Rev. Biochem.*, **48**, 783-836

Hara H., Kaji H. (1987) Random intergration of SV40 in SV40-transformed, immortalized human fibroblasts. *Exp. Cell Res.*, **168**, 531-538

Harlow E., Lane D.P. (1988) *Antibodies: Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York

Hartwell L. (1992) Defects in a cell cycle checkpoint may be responsible for the genomic instability of cancer cells. *Cell*, **71**, 543-546

Hartwell L.H., Weinert T.A. (1989) Checkpoints: controls that ensure the order of cell cycle events. *Science*, **246**, 629-634

Haugen A., Ryberg D., Hansteen I-L., Amstad P. (1990) Neoplastic transformation of a human kidney epithelial cell line transfected with v-Ha-*ras* oncogene. *Int. J. Cancer*, **45**, 572-577

Hayflik L., Moorhead P. (1961) Serial cultivations of human diploid cell strains. *Exp. Cell. Res.*, **25**, 585-621

Hei T.K., Komatsu K., Zaider M. (1988) Oncogenic transformation by charged particles of defined LET. *Carcinogenesis*, **9**, 747-750

Hei T.K., Piao C.Q., Willey J.C., Thomas S., Hall E.J. (1994) Malignant transformation of human bronchial epithelial cells by radon-simulated α-particles. *Carcinogenesis*, **15**, 431-437

Helbig R., Gerland E., Speit G. (1994) The molecular nature of mutations induced by adriamycin at *hprt* locus of V79-4 cells. *Mutagenesis*, **9**, 113-116

Hempelmann L.H., Hall W.J., Phillips M., Cooper R.A., Ames W.R. (1975) Neoplasms in persons treated with X-rays in infancy: fourth survey in 20 years. *J. Natl. Cancer Inst.*, **55**, 519-530

Hieber L., Ponsel G., Ross H., Fenn S., Fromke, Kellerer A.M. (1987) Absence of a dose-rate effect in the transformation of C3H 10T1/2 cells by α-particles. *Int. J. Radiat. Biol.*, **52**, 859-869

Hill C.K., Buonaguro F.M., Myers C.P., Han A., Elkind M.M. (1982) Fission-spectrum neutrons at reduced dose rates enhance neoplastic transformation. *Nature*, **298**, 67-69

Hill C.K., Carnes B.A., Han A., Elkind M.M. (1985) Neoplastic transformation is enhanced by multiple low doses of fission-spectrum neutrons. *Radiat. Res.*, **102**, 404-410

Hollstein M., Sidransky D., Vogelstein B., Harris C.C. (1991) p53 mutation in human cancers. *Science*, **253**, 49-53

Hopkins N., Besmer P., DeLeo A.B., Law L.W. (1981) High-frequency cotransfer of the transformed phenotype and a tumour-specific transplantation antigen by DNA from the 3-methylcholanthrene-induced Meth A sarcoma of BALB/c mice. *Proc. Natl. Acad. Sci. USA*, **78**, 7555-7559

Hutchinson F. (1985) Chemical changes induced in DNA by ionizing radiation. *Progr. Nucleic Acid Res. Mol. Biol.*, **32**, 115-152

Huttermann J., Kohnlein W., Teoule R. (1978) *Effects of Ionizing Radiation on DNA*. Springer-Verlag, Berlin

Ishizaka Y., Itoh F., Tahira T., Ikeda I., Ogura T., Sugimura T., Nagao M. (1989) Presence of aberrant transcripts of *ret* proto-oncogene in a human papillary thyroid carcinoma cell line. *Jpn. J. Cancer Res.*, **80**, 1149-1152

Ito T., Seyama T., Iwamoto K.S., Hayashi T., Mizuno T., Tsuyama N., Dohi K., Nakamura N., Akiyama M. (1993) *In vitro* irradiation is able to cause *RET* oncogene rearrangement. *Cancer Res.*, **53**, 2940-2943

Ito T., Seyama T., Mizuno T., Tsuyama N., Hayashi T., Hayashi Y., Dohi K., Nakamura N., Akiyama M. (1992) Unique association of p53 mutations with undifferentiated but not with differentiated carcinomas of the thyroid gland. *Cancer Res.*, **52**, 1369-1371

Jeffreys A.J., Wilson V., Thein S.L. (1985) Individual-specific 'fingerprints' of human DNA. *Nature*, **316**, 76-79

Jhiang S.M., Caruso D.R., Gilmore E., Ishizaka Y., Tahira T., Nagao M., Shiu I.-M., Mazzaferri E.L. (1992) Detection of the PTC/*ret*^{TPC} oncogene in human thyroid cancers. *Oncogene*, **7**, 1331-1337

Jiang W., Kahn S.M., Guillem J.G., Lu S-H., Weinstein I.B. (1989) Rapid detection of *ras* oncogenes in human tumors: applications to colon, esophageal, and gastric cancer. *Oncogene*, **4**, 923-928

Jones D.S.C., Schofield J.P. (1990) A rapid method for isolating high quality plasmid DNA suitable for DNA sequencing. *Nucleic Acids Res.*, **18**, 7463-7464

Kadhim M.A., MacDonald D.A., Goodhead D.T., Lorimore S.A., Marsden S.J., Wright E.G. (1992) Transmission of chromosomal instability after plutonium α-particle irradiation. *Nature*, **355**, 738-740

Kakunaga T. (1973) A quantitative system for assay of malignant transformation by chemical carcinogens using a clone derived from BALB/3T3. *Int. J. Cancer*, **12**, 463-473

Kakunaga T (1975) Role of cell division in the malignant transformation of mouse cells treated with 3-methylcholanthrene. *Cancer Res.*, **35**, 1635-1642

Kakunaga T. (1978) Neoplastic transformation of human diploid fibroblast cells by chemical carcinogens. *Proc. Natl. Acad. Sci. USA.*, **75**, 1334-1338

Kaplan A.S. (1967) On the natural history of the murine leukemias: presidential address. *Cancer Res.*, **27**, 1325-1340

Kastan M.B., Zhan Q., El-Deiry W.S., Carrier F., Jacks T., Walsh W.V., Plunkett B.S., Vogelstein B., Fornace A.J. (1992) A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in ataxia-telangiectasia. *Cell*, **71**, 587-597

Kazakov V.S., Demidchik E.P., Astakhova L.N. (1992) Thyroid cancer after Chernobyl. *Nature*, **359**, 21

Kennedy A.R., Fox M., Murphy G., Little J.B. (1980) Relationship between X-ray exposure and malignant transformation in C3H 10T1/2 cells. *Proc. Natl. Acad. Sci. USA*, **77**, 7262-7266

Kennedy A.R., Little J.B. (1984) Evidence that a second event in X-ray-induced oncogenic transformation *in vitro* occurs during cellular proliferation. *Radiat. Res.*, **99**, 228-248

Kern S.E., Pietenpol J.A., Thiagalingam S., Seymour A., Kinzler K.W., Vogelstein B. (1992) Oncogenic forms of p53 inhibit p53 regulated gene expression. *Science*, **256**, 827-830

Klein G. (1987) The approaching era of the tumor suppressor genes. *Science*, **238**, 1539-1545

Klein G., Klein E. (1985) Evolution of tumours and the impact of molecular oncology. *Nature*, **315**, 190-195

Kleppe K., Ohsutka E., Kleppe R., Molineux L., Khorana H.G. (1971) Studies on polynucleotides, repair replication of short synthetic DNA's as catalyzed by DNA polymerases. *J. Mol. Biol.*, **56**, 341

Krolewski B., Little J.B. (1989) Molecular analysis of DNA isolated from the different stages of X-ray induced transformation in vitro. *Mol. Carcinogen.*, **2**, 27-33

Krontiris T.G., Cooper G.M. (1981) Transforming activity of human tumor DNAs. *Proc. Natl. Acad. Sci. USA*, **78**, 1181-1184

Kucherlapati R., Hwang S.P., Shimizu N., McDougall J.K., Botchan M.R. (1978) Another chromosomal assignment for a simian virus 40 integration site in human cells. *Proc. Natl. Acad. Sci. USA*, **75**, 4460-4464

Kuerbitz S.J., Plunkett B.S., Walsh W.V., Kastan M.B. (1992) Wild-type p53 is a cell cycle checkpoint determinant following irradiation. *Proc. Natl. Acad. Sci. USA*, **89**, 7491-7495

Lamaire D.G.E., Bothe E., Schulte-Frohlinde D. (1984) Yeilds of radiation-induce main chain scissions of poly U in aqueous solution: strand breakage formation *via* base radicals. *Int. J. Radiat. Biol.*, **45**, 351-358

Land H., Parada L.F., Weinberg R.A. (1983) Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. *Nature*, **304**, 596-602

Lane D.P. (1992) p53, guardian of the genome. *Nature*, **358**, 15-16

Lane D.P, Crawford L.V. (1979) T antigen is bound to a host protein in SV40-transformed cells. *Nature*, **278**, 261-263

LeBeau M., Albain K.S., Larson R.A., Vardiman J.W., Davis E.M., Blough R., Golomb H., Rowley J.D. (1986) Clinical and cytogenetic correlations in sixty-three patients with therapeutic related myelodysplastic syndromes and acute non-lymphocytic leukemia: Further evidence for characteristic abnormalities of chromosomes 5 and 7. *J. Clin. Oncol.*, **4**, 325-345

Lee M., Yang J., Salehi Z., Arnstein P., Chen L., Jay G., Rhim J.S. (1993) Neoplastic transformation of human keratinocytes cell line by the v-*fos* oncogene. *Oncogene*, **8**, 387-393

Lemoine N.R., Mayall E.S., Jones T., Sheer D., McDermid S., Kendall-Taylor P., Wynford-Thomas D. (1989a) Characterisation of human thyroid epithelial cells immortalised *in vitro* by simian virus 40 DNA transfection. *Br. J. Cancer, 60,* 897-903

Lemoine N.R., Mayall E.S., Wyllie F.S., Farr C.J., Hughes D., Anne Padua R., Thurston V., Williams E.D., Wynford-Thomas D. (1988) Activated *ras* oncogenes in human thyroid cancers. *Cancer Res.,* **48,** 4459-4463

Lemoine N.R., Mayall E.S., Wyllie F.S., Williams E.D., Goyns M., Stringer B., Wynford-Thomas D. (1989b) High frequency of *ras* oncogene activation in all stages of human thyroid tumorigenesis. *Oncogene,* **4,** 159-164

Levine A.J. (1990) The p53 protein and its interactions with the oncogene products of the small DNA tumour viruses. *Virology,* **177,** 419-426

Levine A.J., Momand J., Finlay C.A. (1991) The p53 tumour suppressor gene. *Nature,* **351,** 453-456

Levine A.J., Perry M.E., Chang A., Silver A., Dittmer D., Wu M., Welsh D. (1994) The 1993 Walter Hubert lecture: the role of the p53 tumour-suppressor gene in tumorigenesis. *Br. J. Cancer,* **69,** 409-416

Lewin B. (1983) *Gene II.* Oxford University Press, Oxford

Lewin B. (1990) Driving the cell cycle M-phase kinase, its partners, and substrates. *Cell,* **61,** 743-752

Lin F.L., Sperle K., Sternberg N. (1984) Model for homologous recombination during transfer of DNA into mouse cells: Role for DNA ends in the recombination process. *Mol. Cell. Biol.,* **4,** 1020-1034

Lin F.L., Sperle K., Sternberg N. (1987) Extra chromosomal recombination in mammalian cells as studied with single and double strand substrates. *Mol. Cell. Biol.,* **7,** 129-140

Lindsay S., Potter G.D., Chaikoff I.L. (1957) Thyroid neoplasms in rat: A comparison of naturally occuring and I^{131}-induced tumours. *Cancer Res.*, **17**, 183-189

Linzer D.I.H., Levine A.J. (1979) Characterisation of a 54K dalton cellular SV40 tumor antigen present in SV40-transformed cells and uninfected embryonal carcinoma cells. *Cell*, **17**, 43-52

Little J.B. (1977) Radiation carcinogenesis *in vitro*: implication for mechanisms. In: *Origins of Human Cancer*. Hiatt H.H., Watson J.D., Winsten J.A. (eds) Cold Spring Harbor Conferences on Cell Proliferation, Cold Spring Harbor Laboratory, New York, **4**, pp 923-929

Little J.B. (1981) Influence of noncarcinogenic secondary factors on radiation carcinogenesis. *Radiat. Res.*, **87**, 240-250

Little J.B. (1986) Characteristics of radiation-induced neoplastic transformation *in vitro*. *Leuk. Res.*, **10**, 719-725

Little J.B., Su L.N., Kano Y. (1991) Transformation of human diploid fibroblast by radiation and oncogenes. In: *Neoplastic Transformation in Human Cell Culture: Mechanisms of Carcinogenesis*. Rhim J.S., Dritschilo A. (eds) Humana Press, Totowa, NJ, pp 67-79

Livingstone L.R., White A., Sprouse J., Livanos E., Jacks T., Tisty T.D. (1992) Altered cell cycle arrest and gene amplification potential accompany loss of wild-type p53. *Cell*, **70**, 923-935

Ljungquist S., Nindahl T. (1974) A mammalian endonuclease specific for apurinic sites in double-stranded deoxyribonucleic acid. *J. Biol. Chem.*, **249**, 1536-1540

Luna L.G. (1968) *Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology*. McGraw-Hill Book, New York

Maher V.M., Rowan L.A., Silinskas K.C., Kateley S.A., McCormick J.J. (1982) Frequency of UV-induced neoplastic transformation of diploid human fibroblasts is higher in xeroderma pigmentosum cells than in normal cells. *Proc. Natl. Acad. Sci. USA.*, **79**, 2613-2617

Manfredi J.J., Prives C. (1994) The transforming activity of simian virus 40 large tumor antigen. *Biochemica et Biophysica Acta*, **1198**, 65-83

Marshall C.J., Franks L.M., Carbonell A.W. (1977) Markers of neoplastic transformation in epithelial cell lines derived from human carcinomas. *J. Natl. Cancer. Inst.*, **58**, 1743-1747

Martin S.G., Miller R.C., Randers-Pehrson G., Hall E.J. (1993) Radiation-induced neoplastic transformation of human epithelial cells. In: *Proceedings of the International Symposium on Molecular Mechanisma of Radiation and Chemical Carcinogen-Induced Cell Transformation*. pp 12 (Abstract)

Marx J. (1994) A challange to p16 gene as major tumor suppressor. *Science*, **264**, 1846

Masters J.R.W., Bedford P., Kearney A., Povey S., Franks L.M. (1988) Bladder cancer cell line cross-contamination: identification using a locus-specific mini-satellite probe. *Br. J. Cancer*, **57**, 284-286

McCormick J.J., Yang D., Maher V.M., Farber R.A., Neuman W., Peterson W.D., Pollack M.S. (1988) The HuT series of 'carcinogen-transformed' human fibroblast cell lines are derived from the human fibrosarcoma cell line 8387. *Carcinogenesis*, **9**, 2073-2079

Merregaert J., Michels L., van der Rauwelaert E., Lommel L., Golwinkler R., Janowski M. (1986) Oncogene involvment in radiation- and virus-induced mouse osteosarcomas. *Leuk. Res.*, **10**, 915-921

Michalovitz D., Halevy O., Oren M. (1990) Conditional inhibition of transformation and of cell proliferation by a temperature-sensitive mutant of p53. *Cell*, **62**, 671-680

Mietz J.A., Unger T., Huibregtse J.M., Howley P.M. (1992) The transcriptional transactivation function of wild-type p53 is inhibited by SV40 large T-antigen and by HPV-16 E6 oncoprotein. *EMBO J.*, **11**, 5013-5020

Mihara K., Bai L., Kano Y., Miyazaki, Namba M. (1992) Malignant transformation of human fibroblasts previously immortalized with Co-60 gamma rays. *Int. J. Cancer*, **50**, 639-643

Miller R.C., Brenner D.J., Geard C.R., Komatsu K., Marino S.A., Hall E.J. (1988) Oncogenic transformation by fractionated doses of neutrons. *Radiat. Res.*, **114**, 589-598

Miller R.C., Geard C.R., Brenner D.J., Komatsu K., Marino S.A., Hall E.J. (1989) Neutron-energy-dependent oncogenic transformation of C3H 10T1/2 mouse cells. *Radiat. Res.*, **117**, 114-127

Miller R.C., Geard C.R., Geard M.J., Hall E.J. (1992) Cell-cycle-dependent radiation-induced oncogenic transformation of C3H 10T1/2 cells. *Radiat. Res.*, **130**, 129-133

Miller R.C., Hall E.J. (1978) X-ray dose fractionation and oncogenic transformation in cultured mouse embryo cells. *Nature*, **272**, 58-60

Milligan J. R., Aguilera J.A., Ward J.F. (1993a) Variation of single-strand yield with scavanger concentration for plasmid DNA irradiated in aqueous solution. *Radiat. Res.*, **133**, 151-157

Milligan J.R., Aguilera J.A., Ward J.F. (1993b) Variation of single-strand yield with scavanger concentration for SV40 minichromosomes irradiated in aqueous solution. *Radiat. Res.*, **133**, 158-162

Milo G., DiPaolo J.A. (1978) Neoplastic transformation of human diploid cells *in vitro* after chemical carcinogen treatment. *Nature*, **175**, 130-132

Milo G., Noyes I., Donahoe J., Weibrode S. (1981) Neoplastic transformation of human epithelail cells *in vitro* after exposure to chemical carcinogens. *Cancer Res.*, **41**, 5096-5102

Minden M.D., Pawson A.J. (1992) Oncogenes. In: *The Basic Science of Oncology*. Tannock I.F., Hill R.P (eds). McGraw-Hill, Inc., New York, pp 23-40

Modan B., Ron E., Werner A. (1977) Thyroid cancer following scalp irradiation. *Radiology*, **123**, 741-744

Mole R.H. (1958) The dose-response relationship in radiation carcinogenesis. *Brit. M. Bull.*, **14**, 184-189

Muller H.J. (1927) Artificial transmutation of the gene. *Science*, **66**, 84-87

Mullis K., Faloona F. (1987) In: *Methods in enzymology*. Wu R. (ed) Academic Press, New York, **155**, pp 335-346

Murray A.W. (1992) Creative blocks: cell-cycle checkpoints and feedback controls. *Nature*, **359**, 599-604

Namba M., Nishitani K., Fukushima F., Kimoto T., Yuasa Y. (1988) Multi-step neoplastic transformation of normal human fibroblasts by Co-60 gamma rays and Ha-*ras* oncogenes. *Mutatation Res.*, **199**, 415-423

Namba M., Nishitani K., Fyodoh F., Fukushima F., Kimoto T., (1985) Neoplastic transformation of human diploid fibroblasts (KMST-6) by treatment with Co-60 gamma rays. *Int. J. Cancer*, **35**, 275-280

Nigro J.M., Baker J.S., Preisinger A.C., Jessup J.M., Hosteatter R., Cleary K., Bigner S.H., Davidson N., Baylin S., Devilee P., Glover T., Collins F.S., Weston A., Modali R., Harris C.C., Vogelstein B. (1989) Mutations in p53 gene occur in diverse human tumour types. *Nature*, **342**, 705-708

Nusse R. (1986) The activation of cellular oncogenes by retroviral insertion. *Trends Genet.*, **2**, 244-247

O'Brien W., Stenman G., Sager R. (1986) Suppression of tumor growth by senescence in virally transformed human fibroblasts. *Proc. Natl. Acad. Sci. USA*, **83**, 8659-8663

Oleinick N.L.L., Chiu S., Friedman R., Xue and Ramakrishna (1986) DNA protein cross link. New insights into their formation and repair in irradiated mammalian cells. In: *Mechanisms of DNA Damage and Repair*. Simic M.G., Grossman L., Upton A.C. (eds) Plenum Press, New York, pp 181-192

Palle W., Salomaa S. (1994) Radiogenic thyroid cancer in Belarus: Fact of fiction? *J. Radiol. Prot.*, **14**, 265-269

Panet A., Khorana H.G. (1974) The linkage of deoxyribonucleotide template to cellulose and its use in their replication. *Journal of Biological Chemistry*, **249**, 5213-5221

Parkinson F.K., Grabham P., Emmerson A., (1983) A subpopulation of cultural human keratinocytes which is resistant the induction of termal differentialtion related changes by phorbol, 12-myristate, 13-acetate - evidence for an increase in the resistant population following transformation. *Carcinogenesis*, **4**, 857-861

Parsa I., Marsh W.H., Sutton A.L. (1981a) An *in vitro* of human pancreas carcinogenesis: effects of nitroso compounds. *Cancer* (Phila.), **47**, 1543-1551

Parsa I., Marsh W.H., Sutton A.L. (1981b) Effects of dimethylnitrosamine on organ-cultured adult human pancreas. *Am. J. Pathol.*, **102**, 403-411

Parsa I., Bloomfield R.D., Foye C.A., Sutton A.L. (1984) Methylnitrosourea-induced carcinoma in organ-cultured fetal human pancreas. *Cancer Res.*, **44**, 3530-3538

Peehl D.M., Stanbridge E.J. (1981) Anchorage-independent growth of normal human fibroblasts. *Proc. Natl. Acad. Sci. USA*, **78**, 3053-3057

Perucho M., Goldfrab M., Shimizu K., Lama C., Fogh J., Wigler M. (1981) Human-tumor-derived cell lines contain common and different transforming genes. *Cell*, **27**, 467-476

Peto R. (1977) Epidemiology, multistage models, and short term tumorigenesis test. In: *Origins of Human Cancer*. Hiatt M.M., Watson J.D., Winston J.A. (eds), Cold Spring Harbor Laboratory, New York, pp 1403-1428

Pierotti M.A., Santoro M., Jenkins R.B., Sozzi G., Bongarzone I., Grieco M., Monzini N., Miozzo M., Herrmann M.A., Fusco A., Hay I.D., Dalla Porta G., Vecchio G. (1992) Characterization of an inversion on the long arm of chromosome 10 juxtaposing *D10S170* and *RET* and creating the oncogenic sequence *RET/PTC*. *Proc. Natl. Acad. Sci. USA*, **89**, 1616-1620

Pitot H.C. (1981) *Fundamentals of Oncology*. Marcel Dekker, Inc., New York

Potter G.D., Lindsay S., Chaikoff I.L. (1960) Induction of neoplasms in rat thyroid glands by low doses of radioiodine. *Arch. Pathol.*, **69**, 257-269

Powell L.M., Wallis S.C., Pease R.J., Edwards Y.H., Knott T.J., Scott J. (1987) A novel form of tissue-specific RNA processing produces a polipoprotein B48 in intestine. *Cell*, **50**, 831-840

Pulciani S., Santos E., Lauver A.V., Long L.K., Aaronson S.A., Barbicid M. (1982) Oncogenes in solid human tumours. *Nature*, **300**, 539-542

Rawn J.D. (1983) *Biochemistry*. Harper and Row Publishing, New York

Reddel R.R., Ke Y., Kaighn E., Malan-Shibley L., Lechner J.F., Rhim J.S., Harris C.C. (1988) Human bronchial epithelial cells neoplastically transformed by v-Ki-*ras*: altered response to inducers of terminal squamous differentiation. *Oncogene Res.*, **3**, 401-406

Reddy E.P., Reynolds R.K., Santos E., Barbacid M. (1982) A point mutation is responsible for acquisition of transforming properties by the T24 human bladder carcinoma oncogenes. *Nature*, **300**, 149-152

Resnick M.A. (1976) The repair of dsb in DNA: A model involving recombination. *J. Theoretical Biol.*, **59**, 97-106

Reznikoff C.A., Bertram J.S., Brankow D.W., Heidelberger C. (1973a) Quantitative and qualitative studies of chemical transformation of cloned C3H mouse embryo cells sensitive to postconfluence inhibition of cell division. *Cancer Res.*, **33**, 3239-3249

Reznikoff C.A., Brankow D.W., Heidelberger C. (1973b) Establishment and characterization of a cloned line of C3H mouse embryo cells sensitive to postconfluence inhibition of division. *Cancer Res.*, **33**, 3231-3238

Reznikoff C.A., Loretz L.J., Christian B.J., Wu S.-H., Meisner L.F. (1988) Neoplastic transformation of SV40-immortalized human urinary tract epithelial cells by *in vitro* exposure to 3-methylcholanthrene. *Carcinogenesis*, **9**, 1427-1436

Rhim J.S. (1989) Neoplastic transformation of human epithelial cells *in vitro*. *Anticancer Res.*, **9**, 1345-1366

Rhim J.S. (1992) Multistep carcinogenesis and human epithelial cells. In: *Transformation of Human Epithelial Cells*. Milo G.E., Casto B.C., Shuler C.F. (eds). CRC Press, Boca Raton, pp 169-210

Rhim J.S. (1993) Neoplastic transformation of human cells *in vitro*. CRC Press, pp 313-335

Rhim J.S., Dritschilo A. (1991) Neoplastic transformation in human cell systems - an overview. In: *Neoplastic Transformation in Human Cell Culture: Mechanisms of Cracinogenesis*. Rhim J.S., Dritschilo A. (eds) Humana Press, Totowa, NJ, pp xi-xxxi

Rhim J.S., Fujita J., Arnstein P., Aaronson S.A. (1986) Neoplastic conversion of human keratinocytes by adenovirus 12-SV40 virus and chemical carcinogens. *Science*, **323**, 385-388

Rhim J.S., Jay G., Arnstein P., Price F.M., Sanford K.K., Aaronson S.A. (1985) Neoplastic transformation of human epidermal keratinocytes by AD12-SV40 and Kirsten sarcoma virus. *Science*, **227**, 1250-1252

Rhim J.S., Kawakami T., Pierce J., Sanford K., Arnstein P. (1988) Cooperation of v-oncogenes in human epithelial cell transformation. *Leukemia*, **2**, S151-S159

Rhim J.S., Thraves P., Dritschilo A., Kuettel M.R., Lee M.S. (1993) Radiation-induced neoplastic transformation of human cells. *Scanning Microscopy*, **7**, 209-216

Rhim J.S., Trimmer R., Arnstein P., Huebner R.J. (1981) Neoplastic transformation of chimpanzee cells induced by adenovirus type 12-simian virus 40 hybrid virus. *Proc. Natl. Acad. Sci. USA*, **78**, 313-317

Rhim J.S., Yoo J.H., Park J.H., Thraves P., Salehi Z., Dritschilo A. (1990) Evidence for the multistep nature of *in vitro* human epithelial cell carcinogenesis. *Cancer Res.* (Suppl.), **50**, S5653-S5657

Riches A.C., Herceg Z., Bryant P.E., Wynford-Thomas D. (1994) Radiation-induced transformation of SV40-immortalized human thyroid epithelial cells by single and fractionated exposure to gamma-irradiation *in vitro*. *Int. J. Radiat. Biol.*, **66**, 757-765

Riches A.C., Thomas D.B. (1970) The effects of irradiation and anti-lymphocyte serum on the responses to tumour allografts. *Br. J. Cancer*, **24**, 833-842

Riley P.A. (1982) Is the establishment of a clone exibiting defective DNA repair the initial stage of carcinogenesis? *Med. Hypotheses*, **9**, 163-168

Romano J.W., Ehrhart J.C., Duthu A., Kim C.M., Appella E., May P. (1989) Identification and characterization of a p53 gene mutations in a human osteosarcoma cell line. *Oncogene*, **4**, 1483-1488

Ron E., Modava B., Preston E., Alfandary E., Stovall M., Boice J.D. (1989) Thyroid neoplasia following low dose radiation in childhood. *Radiat. Res.*, **120**, 516-531

Rossi H.H., Kellerer A.M. (1974) The validity of risk estimates of leukemia incidence based on Japanese data. *Radiat. Res.*, **58**, 131-140

Rovinski B., Benchimol S. (1988) Immortalization of rat embryo fibroblasts by the cellular p53 oncogene. *Oncogene*, **2**, 445-452

Rowley J.D., Golomb H.M., Vardiman J.W. (1981) Non-random chromosome abnormalities in acute leukemia and dysmielopoietic syndromes in patients with previously treated malignant disease. *Blood*, **58**, 759-767

Saiki R.K., Scharf S., Faloona F., Mullis K.B., Horn G.T., Erlich H.A., Arnheim N. (1985) Enzymatic amplification of b-globin genomic sequences and restriction site analysis for diagnosis of sicle cell anaemia. *Science*, **230**, 1350-1354

Sancar A., Sancar G. B. (1988) DNA repair enzymes. *Ann. Rev. Biochem.*, **57**, 29-67

Sanford K.K., Parshad R. (1991) Deficient DNA repair, an early step in neoplastic transformation of human cells in culture. In: *Neoplastic Transformation in Human Cell Culture: Mechanisms of Carcinogenesis.* Rhim J.S., Dritschilo A. (eds) Humana Press, Totowa, NJ, pp 15-23

Sanford K.K., Parshad R., Gannt R., Tarone R.E., Jones G.M., Price F.M. (1989) Factors affecting and significance of G2 chromatin radiosensitivity in predisposition to cancer. *Int. J. Radiat. Res.*, **55**, 963-981

Sanford K.K., Price F.M., Rhim J.S., Stampfer M.R., Parshad R. (1992) Role of DNA repair in malignant neoplastic transformation of human mammary epithelial cells in culture. *Carcinogenesis*, **13**, 1137-1141

Sankaranarayanan K. (1991) Ionizing radiation and genetic risk. (III) Nature of spontaneous and radiation-induced mutations in mammalian *in vitro* systems and mechanisms of induction of mutations by radiation. *Mutat. Res.*, **258**, 75-97

Sawey M.J., Hood A.T., Burns F.J., Garte S.J. (1987) Activation of c-*myc* and c-K-*ras* oncogenes in primary rat tumors induced by ionizing radiation. *Mol. Cell. Biol.*, **7**, 932-935

Schaeffer W.I. (1983) Usage of vertebrate, invertebrate and plant cell, tissue and organ culture terminology. *Tissue Cult. Rep.*, **17**, 19-23

Schell K., Lane W.T., Casey M.J., Huebner R.J. (1966) Potentiation of oncogenicity of adenovirus type 12 grown in African green monkey kidney cell cultures preinfected with SV40 virus: persistence of both T antigens in the tumors and evidence for possible hybridization. *Proc. Natl. Acad. Sci. USA*, **55**, 81-88

Seeburg P.H., Colby W.W., Capon D.J., Goeddel D.V., Levinson A.D. (1984) Biological properties of human c-Ha-*ras* 1 genes mutated at codon 12. *Nature*, **312**, 71-75

Shigematsu I., Kagan A. (1986) Cancer in atomic bomb survivors. *GANN Monograph on Cancer Research* 32. Japan Scientific Societies Press, Tokyo and Plenum Press, New York

Shih C., Padhy L.C., Murray M., Weinberg R.A. (1981) Transforming genes of carcinomas and neuroblastomas introduced into mouse fibroblasts. *Nature*, **290**, 261-264

Shih C., Shilo B.Z., Goldfarb M.P., Dannenberg R.A., Weinberg R.A. (1979) Passage of phenotypes of chemically transformed cells *via* transfection of DNA and chromatin. *Proc. Natl. Acad. Sci. USA*, **76**, 5714-5718

Shin S., Freedman V.H., Risser R., Pollack R. (1975) Tumorigenicity of virus-transformed cells in *nude* mice is correlated specifically with anchorage independent growth *in vitro*. *Proc. Natl. Acad. Sci. USA*, **72**, 4435-4439

Shuin T., Billings P.C., Lillehaug J.R., Patierno S.R., Roy-Burman P., Landolph J.R. (1986) Enhanced expression of c-*myc* and decreased expression of c-*fos* protooncogenes in chemically and radiation-transformed C3H/10T1/2 Cl 8 mouse embryo cell lines. *Cancer Res.*, **46**, 5302-5311

Silinskas K.C., Kateley S.A., Tower J.E., Maher V.M., McCormick J.J. (1981) Induction of anchorage-independent growth in human fibroblasts by propane sultone. *Cancer Res.*, **41**, 1620-1627

Simpson C.L., Hempelmann L., Fuller L. (1955) Neoplasia in children treated with X-rays in infancy for thymic enlargement. *Radiology*, **64**, 840-845

Small M.B., Gluzman Y., Ozer H.L. (1982) Enhanced transformation of human fibroblasts by origin-defective simian virus 40. *Nature*, **296**, 671-672

Sokal R.R., Rohlf F.J. (1981) *Biometry*. W.H. Freeman and Company, San Francisco

Southern E.M. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.*, **98**, 503-517

Stampfer M.R., Bartley J.C. (1985) Induction of transformation and continuous cell lines from normal human mammary epithelial cells after exposure to benzo[a]pyrene. *Proc. Natl. Acad. Sci. USA*, **82**, 2394-2398

Stiles C.D., Desmond W., Chuman L.M., Sato G., Saier M.H., Jr. (1976) Relationship of cell growth behavior *in vitro* to tumorigenicity in athymic nude mice. *Cancer Res.*, **36**, 3300-3305

Storer J.B. (1982) Radiation carcinogenesis. In: *Cancer: A comprehensive Treatise*. Becker F.F. (ed) Plenum Press, New York, pp 629-659

Sutherland B.M., Cimino J.S., Delihas N., Shih A.G., Oliver R.P. (1980) Ultraviolet light-induced transformation of human cells to anchorage-independent growth. *Cancer Res.*, **40**, 1934-1939

Suzuki M., Watanabe M., Suzuki K., Nakano K., Kaneko I. (1989) Neoplastic cell transformation by heavy ions. *Radiat. Res.*, **120**, 468-476

Sweet B.H., Hillman M.R. (1960) The vacuolating virus. *Proc. Soc. Exp. Biol. Med.*, **105**, 420-427

Tabin C.J., Bradly S.M., Bargmann C.I., Weinberg R.A., Papageorge A.G., Scolnick E.M., Dhar R., Lowy D.R., Chang E.H. (1982) Mechanism of activation of a human oncogene. *Nature*, **300**, 143-149

Tainsky M.A., Cooper C.S., Giovanella B.C., Vande Woude G.F. (1984) An activated *ras* gene: detected in late but not early passage human PA1 teratocarcinoma cells. *Science*, **225**, 643-645

Takahashi T., Nau M.M., Chiba I., Birrer M.J., Rosenberg R.K., Vinocour M., Levitt M., Pass H., Gazdar A.F., Minna J.D. (1989) p53 - a frequent target for genetic abnormalities in lung cancer. *Science*, **246**, 491-494

Taparowski E., Shimizu K., Goldfarb M., Wigler M. (1983) Structure and activation of the human N-*ras* gene. *Cell*, **34**, 581-586

Taparowsky E., Suard Y., Fasano O., Shimizu K., Goldfarb M., Wigler M. (1982) Activation of the T24 bladder carcinoma transforming gene is linked to a single amino acid change. *Nature*, **300**, 762-765

Tejwani R., Witiak D.T., Inbasekaran M.N., Cazar F.D., Milo G.E. (1981) Characteristics of benzo[a]pyrene and A-ring reduced 7,12-dimethyl benz[a]anthracene induced neoplastic transformation of human cells *in vitro*. *Cancer Lett.*, **13**, 119-127

Teoule R. (1987) Radiation-induced DNA damage and its repair. *Int. J. Radiat. Biol.*, **51**, 573-589

Teoule R., Bert C., Bonicel A. (1977) Thymine fragment damage retained in the DNA polynucleotide chain after gamma irradiation in aerated solution. *Radiat. Res.*, **72**, 190-200

Teoule R., Cadet J. (1978) Radiation-induced degradation of the base component in DNA and related substances - final products. In: *Effects of Ionizing on DNA*. Huttermann J., Kohnlein W., Teoule R. (eds) Spring Verlag, Berlin, pp 171-178

Terzaghi M., Little J.B. (1974a) Interaction between radiation and benzo[a]pyrene in an *in vitro* model for malignant transformation. In: *Experimental Lung Cancer. Carcinogenesis and Bioassays*. Karbe E., Park J. (eds) Springer-Verlag, New York, pp 497-506

Terzaghi M., Little J.B. (1974b) Repair of potentially lethal radiation damage is associated with enhancement of malignanat transformation. *Nature*, **253**, 548-549

Terzaghi M., Little J.B. (1976a) X-radiation-induced transformation in a C3H mouse embryo-derived cell line. *Cancer Res.*, **36**, 1367-1374

Terzaghi M., Little J.B. (1976b) Oncogenic transformation *in vitro* after split-dose X-irradiation. *Int. J. Radiat. Biol.*, **29**, 583-587

Thacker J. (1986) The nature of mutants induced by ionizing radiation in cultured hamster cells. III. Molecular characterisation of HPRT-deficient mutant induced by γ-rays or α-particles showing that the majority have deletion of all or part of the *hprt* gene. *Mutat. Res.*, **160**, 267-275

Thacker J., Cox R. (1983) The relationship between specific chromosome aberrations and radiation-induced mutations in cultured mammalian cells. In: *Radiation-Induced Chromosome Damage in Man.* Ishikara T., Sasaki M.S. (eds) Alan R. Liss, New York, pp 235-240

Thraves P.J., Grdina D.J., Rhim J.S., Dritschilo A. (1993) Transformation of human epidermal keratinocytes with fisson neutrons. In: *Proceedings of the Radiation Research Society Meeting.* p 87 (Abstract)

Thraves D., Salehi Z., Dritschilo A., Rhim J.S. (1990) Neoplastic transformation of immortalized human epidermal keratinocytes by ionizing radiation. *Proc. Natl. Acad. Sci. USA,* **87,** 1174-1177

Todaro G.J., Green H. (1963) Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines. *J. Cell Biol.,* **17,** 299-313

(UNSCEAR) United Nations Scientific Committee on the Effects of Atomic Radiation (1964) Report. Supplement 14 (A/5814), United Nations, New York

(UNSCEAR) United Nations. Scientific Committee on the Effects of Atomic Radiation (1977) Sources and Effects of Ionizing Radiation: 1977 report to the General Assembly with annexes. United Nations, New York, 1-725

Upton A.C. (1961) The dose-response relation in radiation-induced cancer. *Cancer Res.,* **21,** 717-729

Upton A.C. (1984) Biological aspects of radiation carcinogenesis. In: *Radiation Carcinogenesis: Epidemiology and Biological Significance.* Boice, J.D. Fraumeni J.F. (eds) Raven Press, New York, pp 9-17

Upton A.C. (1986) Evolving perspectives on the biology and mechanisms of carcinogenesis. *Leuk. Res.,* **10,** 727-734

Urlaub G., Mitchell P.J., Kas E., Chasin L.A., Funange V., Myoda T., Hamlin J. (1986) Effect of gamma rays on the dihydrofolate reductase locus: Deletions and inversions. *Som. Cell Mol. Genet.*, **12**, 555-566

Vahakanagas K.H., Samet J.M., Metcalf R.A., Welsh J.A., Bennett W.P., Lane D.P., Harris C.C. (1992) Mutation of p53 and *ras* genes in radon associated lung cancer from uranium miners. *Lancet*, **339**, 576-580

Varmus H.E. (1982) Form and function of retroviral proviruses. *Science*, **216**, 812-820

Vojtesek B., Bartek J., Midgley C.A., Lane D.P. (1992) An immunochemical analysis of the human nuclear phosphoprotein p53: new monoclonal antibodies and epitope mapping using recombinant p53. *Journal of Immunological Methods*, **151**, 237-244

Vogelstein B., Fearon E.R., Hamilton S.R. et al. (1988) Genetic alterations during colo-rectal tumor development. *New. Engl. J. Med.*, **319**, 525-532

Vousden K.H., Marshall C.J. (1984) Three different activated *ras* genes in mouse tumours; evidence for oncogene activation during progression of a mouse lymphoma. *EMBO J.*, **4**, 913-917

Waldren C., Corsell L., Sognier M.A., Puck T.T. (1986) Measurement of low levels of X-ray mutagenesis in relation to human desease. *Proc. Natl. Acad. Sci. USA*, **83**, 4839-4843

Wallace S.S. (1983) Detection and repair of DNA base damages produced by ionizing radiation. *Env. Mut.*, **5**, 769-788

Ward J.F. (1985) Biochemistry of DNA lesions. *Radiat. Res.* (Suppl.), **104**, S103-S111

Ward J.F., Kuo I. (1976) Strand breaks, base release, and postirradiation changes in DNA γ-irradited in O_2- saturated aqueous solution. *Radiat. Res.*, **66**, 485-

Watson J.D. (1973) *The Molecular Biology of the Gene.* Benjamin Inc. Publishing, New York

Weichselbaum R.R., Beckett M.A., Diamond A.A. (1988) Some retinoblastomas, osteosarcomas and soft tissue sarcomas may share common ethiology. *Proc. Natl. Acad. Sci. USA,* **85,** 2106-2109

Weinberg R. (1989) Oncogenes, antioncogenes and the molecular bases of multistep carcinogenesis. *Cancer Res.,* **49,** 3713-3721

Whaley J.M., Little J.B. (1986) Molecular characterisation of *hprt* mutation induced by low and high LET radiations in human cells. *Mutat. Res.,* **243,** 35-45

Wigler M., Pellicer A., Silverstein S., Axel R., Urlaub G., Chasin L. (1979) DNA-mediated transfer of the adenine phosphoribosyltransferase locus into mammalian cells. *Proc. Natl. Acad. Sci. USA,* **76,** 1373-1376

Willey J.C., Greene J., Bresoud A., Cerutti P., Hei T., Wang N., Maillie D., Cox C., Miles E. (1991) Effects of ionizing radiation on human papillomavirus immortalized human bronchial epilthelial cells. In: *Neoplastic Transformation in Human Cell Culture: Mechanisms of Carcinogenesis.* Rhim J.S, Dritschilo A. (eds) Humana Press, Totowa, NJ, pp 113-117

Willey J.C., Hei T.K., Piao C.Q., Madrid L., Willey J.J., Apostolakos M.J., Hukku B. (1993) Radiation-induced deletion of chromosomal regions containing tumor suppressor genes in human bronchial epithelial cells. *Carcinogenesis,* **14,** 1181-1188

Williams D., Pinchera A., Karaoglou A., Chadwick K.H. (1993) *Thyroid Cancer in Children Living near Chernobyl.* Commission of the European Communities. Expert panel report on the consequences of the Chernobyl accident (EUR 15248 EN). Commission of the European Communities, Luxembourg

Williams G.H., Williams E.D. (1995) Identification of tumour-specific translocations in archival meterial. *J. Pathol.*, **175**, 279-281

Wright P.A., Lemoine N.R., Goretzki P.E., Wyllie F.S., Bond J., Hughes C., Roher H-D., Williams E.D., Wynford-Thomas D. (1991) Mutation of the p53 gene in a differentiated human thyroid carcinoma cell line, but not in primary thyroid tumours. *Oncogene*, **6**, 1693-1697

Wynford-Thomas D., (1991) Oncogenes and anti-oncogenes; the molecular basis of tumour behaviour. *J. Pathol.*, **165**, 187-201

Wynford-Thomas D. (1993) Molecular basis of epithelial tumorigenesis, the thyroid model. *Critical Review in Oncogenesis*, 4, 1-22

Yang C.H., Craise L.M. (1994) Development of human epithelial cell systems for radiation risk assesment. *Adv. Space Res.*, **14**, 115-120

Yang T.C., Craise L.M., Mei M., Tobias C.A. (1985) Neoplastic transformation by heavy charged particles. *Radiat. Res.*, **104**, S177-S187

Yang T.C., Stempfer M.R., Rhim J.S. (1991) Neoplastic transformation of human epithelial cells by ionizing radiation. In: *Neoplastic Transformation in Human Cell Culture: Mechanisms of Carcinogenesis.* Rhim J.S., Dritschilo A. (eds) Humana Press, Totowa, NJ, pp 103-111

Yin Y., Tainsky M.A., Bischoff F.Z., Strong L.C., Wahl G.M. (1992) Wild-type p53 restores cell cycle control and inhibits gene amplification in cells with mutant p53 alleles. *Cell*, **70**, 937-948

Yoakum G.H., Lechner J.F., Gabrielson E.W., Korba B.E., Malan-Shibley L., Willey J.C., Valerio M.G., Shamsuddin A.M., Trump B.F., Harris C.C. (1985) Transformation of human bronchial epithelial cells transfected by Harvey *ras* oncogene. *Science*, **227**, 1174-1179

Yoshida K., Hamatani K., Koide H., Ikeda H., Nakamura N., Akiyama M., Tsuchiyama H., Nakayama E., Shiku H. (1988) Preparation of anti-ras M_r 21,000 protein monoclonal antibodies and immunohistochemical analyses on expression of *ras* genes in human stomach and thyroid cancers. *Cancer Res.*, **48**, 5503-5509

Yuasa Y., Srivastava S.K., Dunn C.Y., Rhim J.S., Reddy E.P., Aaronson S.A. (1983) Acquisition of transforming properties by alternative point mutations with c-*bas* has human proto-oncogene. *Nature*, **303**, 775-779

Zimmerman R.J., Little J.B. (1983a) Characteristics of quantitative assay for the *in vitro* transformation of normal human diploid fibroblasts to anchorage independence by chemical carcinogens. *Cancer Res.*, **43**, 2176-2182

Zimmerman R.J., Little J.B. (1983b) Characteristics of human diploid fibroblasts transformed *in vitro* by chemical carcinogens. *Cancer Res.*, **43**, 2183-2189

Appendix

Appendix I: List of recent publications

Appendix II: Published papers

Recent Publications

Riches A.C., Herceg Z., Bryant P.E., Wynford-Thomas D., Stevens D., Goodhead D. (1995) Transformation of a human thyroid epithelial cell line following exposure to alpha particle or gamma irradiation. *10th International Congress of Radiation Research*. Wurzburg, Germany (Abstract)

Riches A., Herceg Z., Bryant P. (1995) Investigation of *ras* mutations and expression time in radiation-induced oncogenesis using a human thyroid epithelial cell line. Paper presented at the ARR *"Radiation Research 95"*. St Andrews, U.K.

Gamble S., Riches A., Herceg Z., Briscoe T., Bryant P., Colucci S., Arrand J.E. (1995) Incidence of p53 mutation in low dose and low dose-rate irradiated human epithelial cell lines. Paper presented at the ARR *"Radiation Research 95"*. St Andrews, U.K.

Riches A.C., Herceg Z., Bryant P.E., Wynford-Thomas D. (1994) Radiation-induced transformation of SV40-immortalized human thyroid epithelial cells by single and fractionated exposure to gamma-irradiation. *Int. J. Radiat. Biol.*, **66**, 757-765

Riches A.C., Herceg Z., Bryant P.E., Wynford-Thomas D., Stevens D., Goodhead D. (1994) Radiation-induced transformation of a human thyroid cell line after exposure to alpha particle or gamma irradiation. Paper presented at the ARR *"Radiation Research 94"*. Dublin, Ireland

Herceg Z., Bryant P., Riches A. (1993) Radiation-induced transformation of an immortalised human thyroid epithelial cell line. Paper presented at the meeting of the ARR *"Radiation Research 93"*. Guildford, U.K.

Herceg Z., Kljajic R., Horsic E. (1993) Toxicity and radioprotective effects of the radioprotector ethiofos in the pig. *Period. Biol.*, **94**, 257-262.

TRANSFORMATION OF A HUMAN THYROID CELL LINE FOLLOWING EXPOSURE TO ALPHA PARTICLE OR GAMMA IRRADIATION

[1]Riches A.C., [1]Herceg Z., [1]Bryant P.E., [2]Wynford-Thomas D., [3]Stevens D., [3]Goodhead D.

[1]School of Biological & Medical Sciences, University of St. Andrews,
[2]Department of Pathology, University of Wales College of Medicine and
[3]MRC Radiobiology Unit, Chilton, Didcot.

Abstract Transformation of human thyroid epithelial cells has been induced following exposure of these cells to alpha particles from a plutonium 238 source or caesium 137 gamma irradiation. Transformation was assessed by transplantation to athymic nude mice. The tumours were classified as undifferentiated anaplastic carcinomas. Cell lines, produced from the tumours, exhibited a range of radiosensitivities but were in general more sensitive than the parent cell line. The human origin of these cell lines was confirmed by chromosome analysis, immunocytochemical demonstration of specific antigens and DNA fingerprinting. In chemical carcinogenesis with human epithelial cells, the cells have to be passaged to maintain rapid cell growth for several weeks in vitro before transformation can be detected. The expression time after irradiation in vitro was investigated. Tumours were produced following transplantation at 1, 1, 3, and 6 weeks after irradiation. An estimate of the RBE for transformation was 4.

Paper will be presented at the *10th International Congress of Radiation Research*. (September 1995, Wurzburg, Germany)

INVESTIGATION OF *RAS* MUTATIONS AND EXPRESSION TIME IN RADIATION-INDUCED ONCOGENESIS USING A HUMAN THYROID EPITHELIAL CELL LINE

Riches A., Herceg Z., Bryant P.

School of Biological and Medical Sciences, University of St. Andrews

Abstract Following single doses of gamma or alpha particle irradiation, tumours can be induced in a human thyroid epithelial cell line following transplantation to athymic nude mice. The RBE was estimated from cell survival data (RBE 3.9 and 4.8) and from the tumour incidence (RBE 4). Cell lines derived from the tumours have been screened for ras mutations. Following PCR amplification, the products were digested with restriction endonucleases to detect endogenous or primer mediated restriction fragment lenght polymorphisms. No evidence of mutations have been detected in H-ras or K-ras codon 12. In chemical carcinogenesis using human epithelial cell lines, cells have to be passaged following carcinogen treatment before tumours can be detected. Tumours were produced following transplantation at 0, 1, 3 and 6 weeks after gamma irradiation using a human thyroid epithelial cell line.

Paper presented at the ARR *"Radiation Research 95"*. (April, 1995, St Andrews, U.K.)

INCIDENCE OF P53 MUTATION IN LOW DOSE AND LOW DOSE-RATE IRRADIATED HUMAN EPITHELIAL CELL LINES

[1]Gamble S., [2]Riches A., [2]Herceg Z., [2]Briscoe T., [2]Bryant P., [3]Colucci S., [1]Arrand J.E.

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[3]Dept. Physics, Dublin Insitute of Technology, Ireland

Abstract Mutations in the p53 tumour suppressor gene are thought to be involved in 50 to 80% of human tumours, the protein playing a key role in cell cycle control and DNA repair following ionizing radiation damage. To examine the role of p53 mutations in ionizing radiation-mediated, multistage carcinogenesis in human epithelial cells, thyroid (HTori3, p53 normal) were irradiated with a brief single dose of γ or α radiation of between 0.25 and 2 Gy, and normal lung epithelial (L132) cells with 0.5 Gy α (acute dose) at low dose rate (0.01 $Gy \cdot h^{-1}$ to 0.1 $Gy \cdot h^{-1}$). Irradiated HTori3 cells were injected into nude mice, and DNA extracted from the resultant tumours. L132 cells were maintained in culture until the background population died back and areas of dense growth were collected for DNA extraction. SSCP analysis was carried out for p53 exons 5-8. Mutations were detected in HTori3 tumours in exons 5, 7 and 8, but not 6. No correlation has yet been found between mutation and dose or LET of radiation. Sequencing to confirm mutations and SSCP analysis of p53 in L132 cells is ongoing.

Paper presented at the ARR *"Radiation Research 95"*. (April 1995, St Andrews, U.K.

TRANSFORMATION OF A HUMAN THYROID EPITHELIAL CELL LINE FOLLOWING EXPOSURE TO ALPHA PARTICLE OR GAMMA IRRADIATION

[1]Riches A.C., Herceg Z., Bryant P.E., [2]Wynford-Thomas D., [3]Stevens D., Goodhead D. (1995)

[1]School of Biological & Medical Sciences, University of St. Andrews,
[2]Department of Pathology, University of Wales College of Medicine and
[3]MRC Radiobiology Unit, Chilton, Didcot.

Abstract Transformation of human epithelial cells by radiation has proved difficult. A human thyroid epithelial cell line (HTori3) was exposed to single doses of gamma irradiation or alpha particles from a plutonium 238 source, using good track segment irradiation conditions. Tumorigenicity was evaluated following in vitro passaging and transplantation into athymic nude mice. No tumours were observed in the control group while tumours (27/42) were observed in the alpha irradiated (0.125 - 1.5 Gy) groups and in the gamma irradiated (15/19) groups (2 and 4 Gy). This promises to be a useful model for investigating the mechanisms of radiation-induced transformation in human epithelail cells.

Paper presented at the ARR *"Radiation Research 94"*. (1994, Dublin, Ireland)

RADIATION-INDUCED TRANSFORMATION OF AN IMMORTALISED HUMAN THYROID EPITHELIAL CELL LINE

Herceg Z., Bryant P., Riches A.

School of Biological & Medical Sciences, University of St. Andrews,

Abstract Transformation of human epithelial cells with radiation has proved difficult to effect. A human thyroid epithelial cell line wich has been immortalised following transfection with a plasmid containing an defective SV40 genome (HTori-3; Lemoine et al, 1989, Br.J.Cancer 60, 897) has been used for these studies. The cell line is non-tumorigenic in athymic nude mice and expresses human epithelial cytokeratins and SV40 large T protein as revealed by immuocytochemistry. The cells were exposed to single or multiple doses of gamma radiation (^{137}Cs) and passaged after each exposure to maintain continous proliferation of the cells. Cells were then injected subcutaneously into athymic nude mice and screened for tumour formation for 3-6 months. To date one tumour from the 3 x2 Gy group has been observed 17 weeks after transplantation. Explants from the tumour were grown in vitro and passaged cells derived. These cells were tumorigenic in athymic nude mice. The human origin of these cells was confirmed by chromosome analysis and immunocytochemical demonstration of human cytokeratins, large T protein and stabilised p53 protein. The passaged cells clone in agar, have an increased growth rate and are more radiosensitive than the normal epithelial parent cell line. This looks to be a promising approach to studying radiation-induced transformation of human epithelial cells.

Paper presented at the meeting of the ARR *"Radiation Research 93"*. (July 1993, Guildford, U.K.)